(Translation) Mailed: April 15, 2003

NOTIFICATION OF REASONS FOR REJECTION

Patent Application No.: 2000-247729

Examiner's Notice Date: April 8, 2003

Examiner: Keiko Nagai

This application is rejected on the grounds stated below. Any opinion about the rejection must be filed within 60 DAYS of the mailing date hereof.

REASON

The invention is unpatentable under Section 29 (2) of the Patent Law, as being such that the invention could easily have been made by a person with ordinary skill in the art to which the invention pertains, on the basis of the invention described in the following publication(s) distributed in Japan or a foreign country prior to this application.

REMARKS

(1) Claim 1 : Reference 1

Reference 1 discloses a nucleotide sequence of an estrogen receptor gene of medaka fish. Here, it is easily achievable for a person having ordinary skill in the art to prepare a probe based on the nucleotide sequence of the gene discussed in Reference 1, thus obtaining polynucleotide including upstream and downstream regions of the estrogen receptor gene of medaka fish and determining its nucleotide sequence.

(2) Claim 2 : Reference 1

Reference 1 discloses a nucleotide sequence of an estrogen receptor gene of medaka fish. Here, it is easily achievable for a person having ordinary

discussed in Reference 1, thus obtaining an estrogen receptor gene of medaka fish and determining its nucleotide sequence.

(3) Claim 3 : Reference 1

Reference 1 discloses a nucleotide sequence of an estrogen receptor gene of medaka fish, and an amino acid sequence of the estrogen receptor. Here, it is easily achievable for a person having ordinary skill in the art to prepare a probe based on the nucleotide sequence of the gene discussed in Reference 1, thus obtaining an estrogen receptor gene of medaka fish and determining its nucleotide sequence and an amino acid sequence encoded by it.

(4) Claim 4 : Reference 1

As described in remarks (1) and (2) above, it is easily achievable for a person having ordinary skill in the art to obtain polynucleotide recited in Claims 1 and 2. Assembly of a recombinant vector is merely a well-known means.

(5) Claim 5 : Reference 1

Introduction of a gene to a host in order to examine an in-vivo function of a product of an isolated gene is well-known means.

The claims not mentioned in this Official Action are not rejected. If a new reason for rejection is noticed, a further Official Action will be issued.

Reference Cited:

1. Jpn. Pat. Appln. KOKAI Publication 2000-201688

Prior Art Search Report

Searched Field: IPC 7th ed. C12N 15/00
SwissProt/PIR/GeneSeq
Genbank/EMBL/DDBJ/GeneSeq
BIOSIS
MEDLINE
WPIDS

Prior-Art Document(s):

Winn R., Marine Environmental Research, vol. 46 (1-5), p.130 (1998)

pp. 192-199 (1994)

Gray M.A. et al., Environmental Toxicology and Chemistry, vol. 18(11), pp. 2587-2594 (1999)

The result of this prior art search does not constitute the reasons for rejection.

拒絶理由通知書

特許出願の番号	特願２０００－２４７７２９
起案日	平成１５年　４月　８日
特許庁審査官	長井　啓子　　　　　９１２３　４Ｎ００
特許出願人代理人	鈴江　武彦（外　５名）　様
適用条文	第２９条第２項

15. 6. 14

この出願は、次の理由によって拒絶をすべきものである。これについて意見があれば、この通知書の発送の日から６０日以内に意見書を提出して下さい。

理　由

この出願の下記の請求項に係る発明は、その出願前日本国内又は外国において頒布された下記の刊行物に記載された発明に基いて、その出願前にその発明の属する技術の分野における通常の知識を有する者が容易に発明をすることができたものであるから、特許法第２９条第２項の規定により特許を受けることができない。

記　　　（引用文献等については引用文献等一覧参照）

（１）請求項１：引用文献１
引用文献１には、メダカのエストロゲンレセプター遺伝子の塩基配列が開示されている。引用文献１記載の遺伝子の塩基配列を基にしてプローブを作成して、メダカのエストロゲンレセプター遺伝子の上流及び下流の領域を含むポリヌクレオチドを得てその塩基配列を決定することは、当業者が容易になし得る程度のことにすぎない。

（２）請求項２：引用文献１
引用文献１には、メダカのエストロゲンレセプター遺伝子の塩基配列が開示されている。引用文献１記載の遺伝子の塩基配列を基にしてプローブを作成して、メダカのエストロゲンレセプター遺伝子を得てその塩基配列を決定することは、当業者が容易になし得る程度のことにすぎない。

（３）請求項３：引用文献１
引用文献１には、メダカのエストロゲンレセプター遺伝子の塩基酸配列及び当該エストロゲンレセプターのアミノ酸配列が開示されている。引用文献１記載の

広島大学長

遺伝子の塩基配列を基にしてプローブを作成して、メダカのエストロゲンレセプター遺伝子を得てその塩基配列及びそれがコードするアミノ酸配列を決定することは、当業者が容易になし得る程度のことにすぎない。

（４）請求項４：引用文献１
請求項１及び請求項２記載のポリヌクレオチドを得ることが、引用文献１の記載に基づいて当業者が容易になし得たことは、上記（１）及び（２）で説明したとおりである。組み換えベクターを構築することは常套手段にすぎない。

（５）請求項５：引用文献１
単離した遺伝子の産物の生体内機能を探る等の目的で、宿主に遺伝子導入することは、常套手段である。

この拒絶理由通知書中で指摘した請求項以外の請求項に係る発明については、現時点では、拒絶の理由を発見しない。拒絶の理由が新たに発見された場合には拒絶の理由が通知される。

引　用　文　献　等　一　覧

1.特開２０００－２０１６８８号公報

この拒絶理由通知書に不明な点がある場合、または、この案件について面接を希望する場合は、
特許審査第三部生命工学　長井　啓子
Ｔｅｌ．０３－３５８１－１１０１(特許庁代表)
Ｆａｘ．０３－３５０１－０４９１
までご連絡下さい。

－－－－－－－－－－－－－－－－－－－－－－－－－－－－－－－－－－－－－－－

先行技術文献調査結果の記録

・調査した分野　ＩＰＣ第７版　Ｃ１２Ｎ　１５／００
ＳｗｉｓｓＰｒｏｔ／ＰＩＲ／ＧｅｎｅＳｅｑ
Ｇｅｎｂａｎｋ／ＥＭＢＬ／ＤＤＢＪ／ＧｅｎｅＳｅｑ
ＭＥＤＬＩＮＥ
ＷＰＩＤＳ

```
ID   AAA92174 standard; DNA; 1728 BP.
XX
AC   AAA92174;
XX
DT   05-JAN-2001  (first entry)
XX
DE   Oryzias lapites oestrogen receptor encoding DNA SEQ ID NO:2.
XX
KW   Oryzias lapites; oestrogen receptor; ds.
XX
OS   Oryzias lapites.
XX
PN   JP2000201688-A.
XX
PD   25-JUL-2000.
XX
PF   06-APR-1999;    99JP-0098787.
XX
PR   10-NOV-1998;    98JP-0319465.
XX
PA   (SUMO ) SUMITOMO CHEM CO LTD.
XX
DR   WPI; 2000-567950/53.
DR   P-PSDB; AAB20897.
XX
PT   An estrogen receptor gene and its application
XX
PS   Claim 3; Page 11-13; 23pp; Japanese.
XX
CC   The present sequence encodes an oestrogen receptor derived from
CC   Oryzias lapites. Also described are: (1) a vector comprising the
CC   oestrogen receptor gene; (2) a transformant prepared by introducing
CC   the oestrogen receptor gene or vector from (1) into a host cell;
CC   (3) a method for the preparation of an oestrogen receptor comprising
CC   culturing the transformant from (2) to produce the oestrogen receptor;
CC   and (4) a method for the evaluation of oestrogen receptor-activating
CC   ability of a chemical substance in which the chemical substance is
CC   reacted with a transformant prepared by introducing a reporter gene
CC   connected downstream of a transcription controlling region containing
CC   an oestrogen response sequence and the above oestrogen receptor gene to
CC   an oestrogen-nonendogenous host cell. The transformant can be used for
CC   the evaluation of oestrogen receptor-activating ability of a chemical
CC   substance.
XX
SQ   Sequence 1728 BP; 378 A; 514 C; 497 G; 339 T; 0 other;
     atgtaccctg aagagagccg gggttctgga ggggtggctg ctgtggacct tttggaaggg        60
     acgtacgact atgccgcccc caaccctgcc acgactcccc tttacagcca gtccagcacc       120
     ggctactact ctgctcccct ggaaacaaac ggaccccccct cagaaggcag tctgcagtcc      180
     ctgggcagtg ggccgacgag ccctctggtg tttgtgccct ccagccccag actcagtccc       240
     tttatgcatc cacccagcca ccactatctg gaaaccactt ccacgcccgt ttacagatcc       300
     agccaccagg gagcctccag ggaggaccag tgcggctccc gggaggacac gtgcagcctg       360
     ggggagttag gcgccggagc cggggctggg gggtttgaga tggccaaaga cacgcgtttc       420
     tgcgccgtgt gcagcgacta cgcctctggg taccactatg gggtgtggtc ttgtgagggc       480
     tgcaaggcct tcttcaagag gagcatccag ggtcacaatg actatatgtg cccagcgacc       540
     aatcagtgca ctattgacag aaatcgaagg aagggctgtc aggcttgtcg tcttaggaag       600
     tgttacgaag tgggaatgat gaaaggcggt gtgcgcaagg accgcattcg cattttacgg       660
     cgtgacaaac ggcggacagg cgttggtgat ggagacaagg ttgtaaaggg tcaggagcat       720
     aaaacggtgc attatgatgg aaggaaacgc agcagcacag gaggaggagg aggaggagga       780
     ggaggaagac tgtctgtgac cagcatacct cctgagcagg tgctgctcct ccttcagggc       840
     gccgagcccc cgatactctg ctcgcgtcag aagttgagcc gaccgtacac cgaggtcacc       900
     atgatgaccc tgctcaccag catggcagac aaggagctgg tccacatgat cgcctgggcc       960
     aagaagctcc caggttttct gcagctgtcc ctgcacgatc aggtgctgct gctggagagc      1020
     tcgtggctgg aggtgctcat gatcggcctc atttggaggt ccatccactg tcccgggaag      1080
     ctcatctttg cacaagacct catcctggac aggaatgagg gagactgcgt ggaaggcatg      1140
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05-01-2001
P. 1-7 (7)

1 D

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acggagatct tcgacatgct gctggccact gcttcccgct tccgtgtgct caaactcaaa      1200
cctgaggaat tcgtctgcct caaagctatt attttactca actccggtgc tttttctttc      1260
tgcaccggca ccatggagcc acttcacaac agcgcggcgg ttcagagcat gctggacacc      1320
atcacagacg cactcattca ttacatcagt cagtcgggtt acttggccca ggagcaggcg      1380
agacggcagg cccagccgct cctgctgctc tcccacatca ggcacatgag caacaaaggc      1440
atggagcacc tctacagcat gaagtgcaag aacaaagtcc ctctttatga cctcctactg      1500
gagatgctcg atgcccaccg cctgcaccac cccgtcagag cccccagtc cttgtcccaa       1560
gtcgacagag accctccctc caccagcagc ggcgggggtg gaatcgctcc cggttctata      1620
tcagcatctc gaggcagaat cgagagtccg agcagaggcc cctttgctcc cagtgtcctt      1680
cagtatggag ggtcgcgtcc tgactgcacc ccggcccttc aagactga                   1728
//
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>>GSN:AAA92174  Oryzias lapites oestrogen recept  (1728 nt)
 initn: 8586 init1: 8586 opt: 8586  Z-score: 8271.0  bits: 1544.2 E():    0
 99.653% identity (99.653% ungapped) in 1728 nt overlap (211-1938:1-1728)

               190       200       210       220       230       240
EP0111 CGCCTCTCGCCCCGTGACCCCCTCGGTGACATGTACCCTGAAGAGAGCCGGGGTTCTGGA
                                     ::::::::::::::::::::::::::::::
GSN:AA                               ATGTACCCTGAAGAGAGCCGGGGTTCTGGA
                                             10        20        30

               250       260       270       280       290       300
EP0111 GGGGTGGCTGCTGTGGACTTTTTGGAAGGGACGTACGACTATGCCGCCCCCAACCCTGCC
       :::::::::::::::::: :::::::::::::::::::::::::::::::::::::::::
GSN:AA GGGGTGGCTGCTGTGGACCTTTTGGAAGGGACGTACGACTATGCCGCCCCCAACCCTGCC
               40        50        60        70        80        90

               310       320       330       340       350       360
EP0111 ACGACTCCCCTTTACAGCCAGTCCAGCACCGGCTACTACTCTGCTCCCCTGGAAACAAAC
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
GSN:AA ACGACTCCCCTTTACAGCCAGTCCAGCACCGGCTACTACTCTGCTCCCCTGGAAACAAAC
              100       110       120       130       140       150

               370       380       390       400       410       420
EP0111 GGACCCCCCCTCAGAAGGCAGTCTGCAGTCCCTGGGCAGTGGCCGACGAGCCCTCTGGTG
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
GSN:AA GGACCCCCCCTCAGAAGGCAGTCTGCAGTCCCTGGGCAGTGGCCGACGAGCCCTCTGGTG
              160       170       180       190       200       210

               430       440       450       460       470       480
EP0111 TTTGTGCCCTCCAGCCCCAGACTCAGTCCCTTTATGCATCCACCCAGCCACCACTATCTG
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
GSN:AA TTTGTGCCCTCCAGCCCCAGACTCAGTCCCTTTATGCATCCACCCAGCCACCACTATCTG
              220       230       240       250       260       270

               490       500       510       520       530       540
EP0111 GAAACCACTTCCACGCCCGTTTACAGATCCAGCCACCAGGGAGCCTCCAGGGAGGACCAG
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
GSN:AA GAAACCACTTCCACGCCCGTTTACAGATCCAGCCACCAGGGAGCCTCCAGGGAGGACCAG
              280       290       300       310       320       330

               550       560       570       580       590       600
EP0111 TGCGGCTCCCGGGAGGACACGTGCAGCCTGGGGGAGTTAGGCGCCGGAGCCGGGGCTGGG
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
GSN:AA TGCGGCTCCCGGGAGGACACGTGCAGCCTGGGGGAGTTAGGCGCCGGAGCCGGGGCTGGG
              340       350       360       370       380       390

               610       620       630       640       650       660
EP0111 GGGTTTGAGATGGCCAAAGACACGCGTTTCTGCGCCGTGTGCAGCGACTACGCCTCTGGG
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
GSN:AA GGGTTTGAGATGGCCAAAGACACGCGTTTCTGCGCCGTGTGCAGCGACTACGCCTCTGGG
              400       410       420       430       440       450

               670       680       690       700       710       720
EP0111 TACCACTATGGGGTGTGGTCTTGTGAGGGCTGCAAGGCCTTCTTCAAGAGGAGCATCCAG
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
GSN:AA TACCACTATGGGGTGTGGTCTTGTGAGGGCTGCAAGGCCTTCTTCAAGAGGAGCATCCAG
              460       470       480       490       500       510

               730       740       750       760       770       780
EP0111 GGTCACAATGACTATATGTGCCCAGCGACCAATCAGTGCACTATTGACAGAAATCGGAGG
       :::::::::::::::::::::::::::::::::::::::::::::::::::::::: :::
GSN:AA GGTCACAATGACTATATGTGCCCAGCGACCAATCAGTGCACTATTGACAGAAATCGAAGG
              520       530       540       550       560       570
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              790       800       810       820       830       840
EP0111 AAGAGCTGCCAGGCTTGTCGTCTTAGGAAGTGTTACGAAGTGGGAATGATGAAAGGCGGT
       ::: :::: ::::::::::::::::::::::::::::::::::::::::::::::::::
GSN:AA AAGGGCTGTCAGGCTTGTCGTCTTAGGAAGTGTTACGAAGTGGGAATGATGAAAGGCGGT
              580       590       600       610       620       630

              850       860       870       880       890       900
EP0111 GTGCGCAAGGACCGCATTCGCATTTTACGGCGTGACAAACGGCGGACAGGCGTTGGTGAT
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
GSN:AA GTGCGCAAGGACCGCATTCGCATTTTACGGCGTGACAAACGGCGGACAGGCGTTGGTGAT
              640       650       660       670       680       690

              910       920       930       940       950       960
EP0111 GGAGACAAGGTTGTAAAGGGTCAGGAGCATAAAACGGTGCATTATGATGGAAGGAAACGC
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
GSN:AA GGAGACAAGGTTGTAAAGGGTCAGGAGCATAAAACGGTGCATTATGATGGAAGGAAACGC
              700       710       720       730       740       750

              970       980       990      1000      1010      1020
EP0111 AGCAGCACAGGAGGAGGAGGAGGAGGAGGAGGAGGAAGACTGTCTGTGACCAGCATACCT
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
GSN:AA AGCAGCACAGGAGGAGGAGGAGGAGGAGGAGGAGGAAGACTGTCTGTGACCAGCATACCT
              760       770       780       790       800       810

             1030      1040      1050      1060      1070      1080
EP0111 CCTGAGCAGGTGCTGCTCCTCCTTCAGGGCGCCGAGCCCCCGATACTCTGCTCGCGTCAG
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
GSN:AA CCTGAGCAGGTGCTGCTCCTCCTTCAGGGCGCCGAGCCCCCGATACTCTGCTCGCGTCAG
              820       830       840       850       860       870

             1090      1100      1110      1120      1130      1140
EP0111 AAGTTGAGCCGACCGTACACCGAGGTCACCATGATGACCCTGCTCACCAGCATGGCAGAC
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
GSN:AA AAGTTGAGCCGACCGTACACCGAGGTCACCATGATGACCCTGCTCACCAGCATGGCAGAC
              880       890       900       910       920       930

             1150      1160      1170      1180      1190      1200
EP0111 AAGGAGCTGGTCCACATGATCGCCTGGGCCAAGAAGCTCCCAGGTTTTCTGCAGCTGTCC
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
GSN:AA AAGGAGCTGGTCCACATGATCGCCTGGGCCAAGAAGCTCCCAGGTTTTCTGCAGCTGTCC
              940       950       960       970       980       990

             1210      1220      1230      1240      1250      1260
EP0111 CTGCACGATCAGGTGCTGCTGCTGGAGAGCTCGTGGCTGGAGGTGCTCATGATCGGCCTC
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
GSN:AA CTGCACGATCAGGTGCTGCTGCTGGAGAGCTCGTGGCTGGAGGTGCTCATGATCGGCCTC
             1000      1010      1020      1030      1040      1050

             1270      1280      1290      1300      1310      1320
EP0111 ATTTGGAGGTCCATCCACTGTCCCGGGAAGCTCATCTTTGCACAAGACCTCATCCTGGAC
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
GSN:AA ATTTGGAGGTCCATCCACTGTCCCGGGAAGCTCATCTTTGCACAAGACCTCATCCTGGAC
             1060      1070      1080      1090      1100      1110

             1330      1340      1350      1360      1370      1380
EP0111 AGGAATGAGGGAGACTGCGTGGAAGGCATGACGGAGATCTTCGACATGCTGCTGGCCACT
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
GSN:AA AGGAATGAGGGAGACTGCGTGGAAGGCATGACGGAGATCTTCGACATGCTGCTGGCCACT
             1120      1130      1140      1150      1160      1170

             1390      1400      1410      1420      1430      1440
EP0111 GCTTCCCGCTTCCGTGTGCTCAAACTCAAACCTGAGGAATTCGTCTGCCTCAAAGCTATT
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
GSN:AA GCTTCCCGCTTCCGTGTGCTCAAACTCAAACCTGAGGAATTCGTCTGCCTCAAAGCTATT
             1180      1190      1200      1210      1220      1230
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            1450      1460      1470      1480      1490      1500
EP0111 ATTTTACTCAACTCCGGTGCTTTTTCTTTCTGCACCGGCACCATGGAGCCACTTCACAAC
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
GSN:AA ATTTTACTCAACTCCGGTGCTTTTTCTTTCTGCACCGGCACCATGGAGCCACTTCACAAC
            1240      1250      1260      1270      1280      1290

            1510      1520      1530      1540      1550      1560
EP0111 AGCGCGGCGGTTCAGAGCATGCTGGACACCATCACAGACGCACTCATTCATTACATCAGT
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
GSN:AA AGCGCGGCGGTTCAGAGCATGCTGGACACCATCACAGACGCACTCATTCATTACATCAGT
            1300      1310      1320      1330      1340      1350

            1570      1580      1590      1600      1610      1620
EP0111 CAGTCGGGTTACTTGGCCCAGGAGCAGGCGAGACGGCAGGCCCAGCTGCTCCTGCTGCTC
       :::::::::::::::::::::::::::::::::::::::::::::: :::::::::::::
GSN:AA CAGTCGGGTTACTTGGCCCAGGAGCAGGCGAGACGGCAGGCCCAGCCGCTCCTGCTGCTC
            1360      1370      1380      1390      1400      1410

            1630      1640      1650      1660      1670      1680
EP0111 TCCCACATCAGGCACATGAGCAACAAAGGCATGGAGCACCTCTACAGCATGAAGTGCAAG
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
GSN:AA TCCCACATCAGGCACATGAGCAACAAAGGCATGGAGCACCTCTACAGCATGAAGTGCAAG
            1420      1430      1440      1450      1460      1470

            1690      1700      1710      1720      1730      1740
EP0111 AACAAAGTCCCTCTTTATGACCTCCTACTGGAGATGCTCGATGCCCACCGCCTGCACCAC
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
GSN:AA AACAAAGTCCCTCTTTATGACCTCCTACTGGAGATGCTCGATGCCCACCGCCTGCACCAC
            1480      1490      1500      1510      1520      1530

            1750      1760      1770      1780      1790      1800
EP0111 CCCGTCAGAGCACCCCAGTCCTTGTCCCAAGTCGACAGAGACCCTCCCTCCACCAGCAGC
       ::::::::::: ::::::::::::::::::::::::::::::::::::::::::::::::
GSN:AA CCCGTCAGAGCCCCCCAGTCCTTGTCCCAAGTCGACAGAGACCCTCCCTCCACCAGCAGC
            1540      1550      1560      1570      1580      1590

            1810      1820      1830      1840      1850      1860
EP0111 GGCGGGGGTGGAATCGCTCCCGGTTCTATATCAGCATCTCGAGGCAGAATCGAGAGTCCG
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
GSN:AA GGCGGGGGTGGAATCGCTCCCGGTTCTATATCAGCATCTCGAGGCAGAATCGAGAGTCCG
            1600      1610      1620      1630      1640      1650

            1870      1880      1890      1900      1910      1920
EP0111 AGCAGAGGCCCCTTTGCTCCCAGTGTCCTTCAGTATGGAGGGTCGCGTCCTGACTGCACC
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
GSN:AA AGCAGAGGCCCCTTTGCTCCCAGTGTCCTTCAGTATGGAGGGTCGCGTCCTGACTGCACC
            1660      1670      1680      1690      1700      1710

            1930      1940      1950      1960      1970      1980
EP0111 CCGGCCCTTCAAGACTGAGCACACAGTCCAAGGCCCTTTTTTTGTGGCTCAAGGGTTCAG
       ::::::::::::::::::
GSN:AA CCGGCCCTTCAAGACTGA
            1720
```

1Y

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ID   AAB20897 standard; Protein; 575 AA.
XX
AC   AAB20897;
XX
DT   05-JAN-2001  (first entry)
XX
DE   Oryzias lapites oestrogen receptor protein SEQ ID NO:1.
XX
KW   Oryzias lapites; oestrogen receptor.
XX
OS   Oryzias lapites.
XX
PN   JP2000201688-A.
XX
PD   25-JUL-2000.
XX
PF   06-APR-1999;   99JP-0098787.
XX
PR   10-NOV-1998;   98JP-0319465.
XX
PA   (SUMO ) SUMITOMO CHEM CO LTD.
XX
DR   WPI; 2000-567950/53.
DR   N-PSDB; AAA92174.
XX
PT   An estrogen receptor gene and its application  -
XX
PS   Claim 1; Page 9-10; 23pp; Japanese.
XX
CC   The present sequence represents an oestrogen receptor derived from
CC   Oryzias lapites. Also described are: (1) a vector comprising the
CC   oestrogen receptor gene; (2) a transformant prepared by introducing
CC   the oestrogen receptor gene or vector from (1) into a host cell;
CC   (3) a method for the preparation of an oestrogen receptor comprising
CC   culturing the transformant from (2) to produce the oestrogen receptor;
CC   and (4) a method for the evaluation of oestrogen receptor-activating
CC   ability of a chemical substance in which the chemical substance is
CC   reacted with a transformant prepared by introducing a reporter gene
CC   connected downstream of a transcription controlling region containing
CC   an oestrogen response sequence and the above oestrogen receptor gene to
CC   an oestrogen-nonendogenous host cell. The transformant can be used for
CC   the evaluation of oestrogen receptor-activating ability of a chemical
CC   substance.
XX
SQ   Sequence   575 AA;
SQ   37 A; 38 R; 10 N; 27 D; 0  B; 18 C; 24 Q; 29 E; 0  Z; 58 G; 19 H;
SQ   22 I; 61 L; 24 K; 19 M; 14 F; 37 P; 58 S; 29 T; 4  W; 20 Y; 27 V;
SQ   0 Others;
     mypeesrgsg gvaavdlleg tydyaapnpa ttplysqsst gyysapletn gppsegslqs
     lgsqptsplv fvpssprlsp fmhppshhyl ettstpvyrs shqgasredq cgsredtcsl
     gelgagagag gfemakdtrf cavcsdyasg yhygvwsceg ckaffkrsiq ghndymcpat
     nqctidrnrr kgcqacrlrk cyevgmmkgg vrkdririlr rdkrrtgvqd gdkvvkqqeh
     ktvhydgrkr sstggggggg ggrlsvtsip peqvllllqg aeppilcsrq klsrpytevt
     mmtlltsmad kelvhmiawa kklpgflqls lhdqvllles swlevlmigl iwrsihcpgk
     lifaqdlild rnegdcvegm teifdmllat asrfrvlklk peefvclkai illnsgafsf
     ctgtmeplhn saavqsmldt itdalihyis qsgylaqeqa rrqaqpllll shirhmsnkg
     mehlysmkck nkvplydlll emldahrlhh pvrapqslsq vdrdppstss ggggiapgsi
     sasrgriesp srgpfapsvl qyggsrpdct palqd
//
```

```
>>GSP:AAB20897  Oryzias lapites oestrogen recept  (575 aa)
 initn: 3905 init1: 3905 opt: 3905  Z-score: 2879.0  bits: 544.9 E(): 2.1e-152
Smith-Waterman score: 3905;  99.478% identity (99.478% ungapped) in 575 aa overlap
(210-1934:1-575)

     210       240       270       300       330       360
EP0111 MYPEESRGSGGVAAVDFLEGTYDYAAPNPATTPLYSQSSTGYYSAPLETNGPPSEGSLQS
       ::::::::::::::::.:::::::::::::::::::::::::::::::::::::::::::
GSP:AA MYPEESRGSGGVAAVDLLEGTYDYAAPNPATTPLYSQSSTGYYSAPLETNGPPSEGSLQS
               10        20        30        40        50        60

     390       420       450       480       510       540
EP0111 LGSGPTSPLVFVPSSPRLSPFMHPPSHHYLETTSTPVYRSSHQGASREDQCGSREDTCSL
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
GSP:AA LGSGPTSPLVFVPSSPRLSPFMHPPSHHYLETTSTPVYRSSHQGASREDQCGSREDTCSL
               70        80        90       100       110       120

     570       600       630       660       690       720
EP0111 GELGAGAGAGGFEMAKDTRFCAVCSDYASGYHYGVWSCEGCKAFFKRSIQGHNDYMCPAT
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
GSP:AA GELGAGAGAGGFEMAKDTRFCAVCSDYASGYHYGVWSCEGCKAFFKRSIQGHNDYMCPAT
              130       140       150       160       170       180

     750       780       810       840       870       900
EP0111 NQCTIDRNRRKSCQACRLRKCYEVGMMKGGVRKDRIRILRRDKRRTGVGDGDKVVKGQEH
       :::::::::::.::::::::::::::::::::::::::::::::::::::::::::::::
GSP:AA NQCTIDRNRRKGCQACRLRKCYEVGMMKGGVRKDRIRILRRDKRRTGVGDGDKVVKGQEH
              190       200       210       220       230       240

     930       960       990      1020      1050      1080
EP0111 KTVHYDGRKRSSTGGGGGGGGGRLSVTSIPPEQVLLLLQGAEPPILCSRQKLSRPYTEVT
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
GSP:AA KTVHYDGRKRSSTGGGGGGGGGRLSVTSIPPEQVLLLLQGAEPPILCSRQKLSRPYTEVT
              250       260       270       280       290       300

    1110      1140      1170      1200      1230      1260
EP0111 MMTLLTSMADKELVHMIAWAKKLPGFLQLSLHDQVLLLESSWLEVLMIGLIWRSIHCPGK
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
GSP:AA MMTLLTSMADKELVHMIAWAKKLPGFLQLSLHDQVLLLESSWLEVLMIGLIWRSIHCPGK
              310       320       330       340       350       360

    1290      1320      1350      1380      1410      1440
EP0111 LIFAQDLILDRNEGDCVEGMTEIFDMLLATASRFRVLKLKPEEFVCLKAIILLNSGAFSF
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
GSP:AA LIFAQDLILDRNEGDCVEGMTEIFDMLLATASRFRVLKLKPEEFVCLKAIILLNSGAFSF
              370       380       390       400       410       420

    1470      1500      1530      1560      1590      1620
EP0111 CTGTMEPLHNSAAVQSMLDTITDALIHYISQSGYLAQEQARRQAQLLLLSHIRHMSNKG
       ::::::::::::::::::::::::::::::::::::::::::::: ::::::::::::::
GSP:AA CTGTMEPLHNSAAVQSMLDTITDALIHYISQSGYLAQEQARRQAQPLLLLSHIRHMSNKG
              430       440       450       460       470       480

    1650      1680      1710      1740      1770      1800
EP0111 MEHLYSMKCKNKVPLYDLLLEMLDAHRLHHPVRAPQSLSQVDRDPPSTSSGGGGIAPGSI
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
GSP:AA MEHLYSMKCKNKVPLYDLLLEMLDAHRLHHPVRAPQSLSQVDRDPPSTSSGGGGIAPGSI
              490       500       510       520       530       540

    1830      1860      1890      1920
EP0111 SASRGRIESPSRGPFAPSVLQYGGSRPDCTPALQD
       :::::::::::::::::::::::::::::::::::
GSP:AA SASRGRIESPSRGPFAPSVLQYGGSRPDCTPALQD
              550       560       570
```

P.D. 05-01-2001
P. 1-9
9

1 D

```
ID   AAA92175 standard; DNA; 1863 BP.
XX
AC   AAA92175;
XX
DT   05-JAN-2001  (first entry)
XX
DE   Oryzias lapites oestrogen receptor encoding DNA SEQ ID NO:4.
XX
KW   Oryzias lapites; oestrogen receptor; ds.
XX
OS   Oryzias lapites.
XX
PN   JP2000201688-A.
XX
PD   25-JUL-2000.
XX
PF   06-APR-1999;   99JP-0098787.
XX
PR   10-NOV-1998;   98JP-0319465.
XX
PA   (SUMO ) SUMITOMO CHEM CO LTD.
XX
DR   WPI; 2000-567950/53.
DR   P-PSDB; AAB20898.
XX
PT   An estrogen receptor gene and its application.
XX
PS   Claim 4; Page 15-17; 23pp; Japanese.
XX
CC   The present sequence encodes an oestrogen receptor derived from
CC   Oryzias lapites. Also described are: (1) a vector comprising the
CC   oestrogen receptor gene; (2) a transformant prepared by introducing
CC   the oestrogen receptor gene or vector from (1) into a host cell;
CC   (3) a method for the preparation of an oestrogen receptor comprising
CC   culturing the transformant from (2) to produce the oestrogen receptor;
CC   and (4) a method for the evaluation of oestrogen receptor-activating
CC   ability of a chemical substance in which the chemical substance is
CC   reacted with a transformant prepared by introducing a reporter gene
CC   connected downstream of a transcription controlling region containing
CC   an oestrogen response sequence and the above oestrogen receptor gene to
CC   an oestrogen-nonendogenous host cell. The transformant can be used for
CC   the evaluation of oestrogen receptor-activating ability of a chemical
CC   substance.
XX
SQ   Sequence 1863 BP; 406 A; 565 C; 531 G; 361 T; 0 other;
     atgagtaaga gacagagctc ggtgcagatc aggcagctgt tcggaccagc actcagatcc        60
     aggatcagcc cagcctcctc agagctggag accctctccc cacctcgcct ctcgccccgt       120
     gacccccctcg gtgacatgta ccctgaagag agccggggtt ctggaggggt ggctgctgtg      180
     gacctttgg aagggacgta cgactatgcc gcccccaacc ctgccacgac tccccttac         240
     agccagtcca gcaccggcta ctactctgct cccctggaaa caaacggacc cccctcagaa       300
     ggcagtctgc agtccctggg cagtgggccg acgagccctc tggtgtttgt gccctccagc       360
     cccagactca gtccctttat gcatccaccc agccaccact atctggaaac cacttccacg       420
     cccgtttaca gatccagcca ccagggagcc tccagggagg accagtgcgg ctcccgggag       480
     gacacgtgca gcctggggga gttaggcgcc ggagccgggg ctggggggtt tgagatggcc       540
     aaagacacgc gtttctgcgc cgtgtgcagc gactacgcct ctgggtacca ctatggggtg       600
     tggtcttgtg agggctgcaa ggccttcttc aagaggagca tccagggtca caatgactat       660
     atgtgcccag cgaccaatca gtgcactatt gacagaaatc gaaggaaggg ctgtcaggct       720
     tgtcgtctta ggaagtgtta cgaagtggga atgatgaaag gcggtgtgcg caaggaccgc       780
     attcgcattt tacggcgtga caaacggcgg acaggcgttg gtgatggaga caaggttgta       840
     aagggtcagg agcataaaac ggtgcattat gatggaagga aacgcagcag cacaggagga       900
     ggaggaggag gaggaggagg aagactgtct gtgaccagca tacctcctga gcaggtgctg       960
     ctcctccttc agggcgccga gcccccgata ctctgctcgc gtcagaagtt gagccgaccg      1020
     tacaccgagg tcaccatgat gaccctgctc accagcatgg cagacaagga gctggtccac      1080
     atgatcgcct gggccaagaa gctcccaggt tttctgcagc tgtccctgca cgatcaggtg      1140
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XP-002181483

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ctgctgctgg agagctcgtg gctggaggtg ctcatgatcg gcctcatttg gaggtccatc 1200
cactgtcccg ggaagctcat ctttgcacaa gacctcatcc tggacaggaa tgagggagac 1260
tgcgtggaag gcatgacgga gatcttcgac atgctgctgg ccactgcttc ccgcttccgt 1320
gtgctcaaac tcaaacctga ggaattcgtc tgcctcaaag ctattatttt actcaactcc 1380
ggtgcttttt ctttctgcac cggcaccatg gagccacttc acaacagcgc ggcggttcag 1440
agcatgctgg acaccatcac agacgcactc attcattaca tcagtcagtc gggttacttg 1500
gcccaggagc aggcgagacg gcaggcccag ccgctcctgc tgctctccca catcaggcac 1560
atgagcaaca aaggcatgga gcacctctac agcatgaagt gcaagaacaa agtccctctt 1620
tatgacctcc tactggagat gctcgatgcc caccgcctgc accaccccgt cagagccccc 1680
cagtccttgt cccaagtcga cagagaccct ccctccacca gcagcggcgg gggtggaatc 1740
gctcccggtt ctatatcagc atctcgaggc agaatcgaga gtccgagcag aggccccttt 1800
gctcccagtg tccttcagta tggagggtcg cgtcctgact gcaccccggc ccttcaagac 1860
tga 1863
//
```

```
>>GSN:AAA92175 Oryzias lapites oestrogen recept (1863 nt)
 initn: 9261 init1: 9261 opt: 9261  Z-score: 8922.0  bits: 1664.7 E():    0
 99.678% identity (99.678% ungapped) in 1863 nt overlap (76-1938:1-1863)

          50        60        70        80        90       100
EP0111 CGTGTTGCGCAGCACATCTGAGGATGATTCATGAGTAAGAGACAGAGCTCGGTGCAGATC
                                     ::::::::::::::::::::::::::::::
GSN:AA                               ATGAGTAAGAGACAGAGCTCGGTGCAGATC
                                             10        20        30

         110       120       130       140       150       160
EP0111 AGGCAGCTGTTCGGACCAGCACTCAGATCCAGGATCAGCCCAGCCTCCTCAGAGCTGGAG
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
GSN:AA AGGCAGCTGTTCGGACCAGCACTCAGATCCAGGATCAGCCCAGCCTCCTCAGAGCTGGAG
               40        50        60        70        80        90

         170       180       190       200       210       220
EP0111 ACCCTCTCCCCACCTCGCCTCTCGCCCCGTGACCCCCTCGGTGACATGTACCCTGAAGAG
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
GSN:AA ACCCTCTCCCCACCTCGCCTCTCGCCCCGTGACCCCCTCGGTGACATGTACCCTGAAGAG
              100       110       120       130       140       150

         230       240       250       260       270       280
EP0111 AGCCGGGGTTCTGGAGGGGTGGCTGCTGTGGACTTTTTGGAAGGGACGTACGACTATGCC
       ::::::::::::::::::::::::::::::::: ::::::::::::::::::::::::::
GSN:AA AGCCGGGGTTCTGGAGGGGTGGCTGCTGTGGACCTTTTGGAAGGGACGTACGACTATGCC
              160       170       180       190       200       210

         290       300       310       320       330       340
EP0111 GCCCCCAACCCTGCCACGACTCCCCTTTACAGCCAGTCCAGCACCGGCTACTACTCTGCT
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
GSN:AA GCCCCCAACCCTGCCACGACTCCCCTTTACAGCCAGTCCAGCACCGGCTACTACTCTGCT
              220       230       240       250       260       270

         350       360       370       380       390       400
EP0111 CCCCTGGAAACAAACGGACCCCCCTCAGAAGGCAGTCTGCAGTCCCTGGGCAGTGGGCCG
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
GSN:AA CCCCTGGAAACAAACGGACCCCCCTCAGAAGGCAGTCTGCAGTCCCTGGGCAGTGGGCCG
              280       290       300       310       320       330

         410       420       430       440       450       460
EP0111 ACGAGCCCTCTGGTGTTTGTGCCCTCCAGCCCCAGACTCAGTCCCTTTATGCATCCACCC
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
GSN:AA ACGAGCCCTCTGGTGTTTGTGCCCTCCAGCCCCAGACTCAGTCCCTTTATGCATCCACCC
              340       350       360       370       380       390

         470       480       490       500       510       520
EP0111 AGCCACCACTATCTGGAAACCACTTCCACGCCCGTTTACAGATCCAGCCACCAGGGAGCC
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
GSN:AA AGCCACCACTATCTGGAAACCACTTCCACGCCCGTTTACAGATCCAGCCACCAGGGAGCC
              400       410       420       430       440       450

         530       540       550       560       570       580
EP0111 TCCAGGGAGGACCAGTGCGGCTCCCGGGAGGACACGTGCAGCCTGGGGGAGTTAGGCGCC
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
GSN:AA TCCAGGGAGGACCAGTGCGGCTCCCGGGAGGACACGTGCAGCCTGGGGGAGTTAGGCGCC
              460       470       480       490       500       510

         590       600       610       620       630       640
EP0111 GGAGCCGGGGCTGGGGGGTTTGAGATGGCCAAAGACACGCGTTTCTGCGCCGTGTGCAGC
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
GSN:AA GGAGCCGGGGCTGGGGGGTTTGAGATGGCCAAAGACACGCGTTTCTGCGCCGTGTGCAGC
              520       530       540       550       560       570

         650       660       670       680       690       700
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EP0111 GACTACGCCTCTGGGTACCACTATGGGGTGTGGTCTTGTGAGGGCTGCAAGGCCTTCTTC
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
GSN:AA GACTACGCCTCTGGGTACCACTATGGGGTGTGGTCTTGTGAGGGCTGCAAGGCCTTCTTC
              580       590       600       610       620       630

         710       720       730       740       750       760
EP0111 AAGAGGAGCATCCAGGGTCACAATGACTATATGTGCCCAGCGACCAATCAGTGCACTATT
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
GSN:AA AAGAGGAGCATCCAGGGTCACAATGACTATATGTGCCCAGCGACCAATCAGTGCACTATT
              640       650       660       670       680       690

         770       780       790       800       810       820
EP0111 GACAGAAATCGGAGGAAGAGCTGCCAGGCTTGTCGTCTTAGGAAGTGTTACGAAGTGGGA
       ::::::::::: :::::: :::: ::::::::::::::::::::::::::::::::::::
GSN:AA GACAGAAATCGAAGGAAGGGCTGTCAGGCTTGTCGTCTTAGGAAGTGTTACGAAGTGGGA
              700       710       720       730       740       750

         830       840       850       860       870       880
EP0111 ATGATGAAAGGCGGTGTGCGCAAGGACCGCATTCGCATTTTACGGCGTGACAAACGGCGG
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
GSN:AA ATGATGAAAGGCGGTGTGCGCAAGGACCGCATTCGCATTTTACGGCGTGACAAACGGCGG
              760       770       780       790       800       810

         890       900       910       920       930       940
EP0111 ACAGGCGTTGGTGATGGAGACAAGGTTGTAAAGGGTCAGGAGCATAAAACGGTGCATTAT
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
GSN:AA ACAGGCGTTGGTGATGGAGACAAGGTTGTAAAGGGTCAGGAGCATAAAACGGTGCATTAT
              820       830       840       850       860       870

         950       960       970       980       990      1000
EP0111 GATGGAAGGAAACGCAGCAGCACAGGAGGAGGAGGAGGAGGAGGAGGAGGAAGACTGTCT
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
GSN:AA GATGGAAGGAAACGCAGCAGCACAGGAGGAGGAGGAGGAGGAGGAGGAGGAAGACTGTCT
              880       890       900       910       920       930

        1010      1020      1030      1040      1050      1060
EP0111 GTGACCAGCATACCTCCTGAGCAGGTGCTGCTCCTCCTTCAGGGCGCCGAGCCCCCGATA
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
GSN:AA GTGACCAGCATACCTCCTGAGCAGGTGCTGCTCCTCCTTCAGGGCGCCGAGCCCCCGATA
              940       950       960       970       980       990

        1070      1080      1090      1100      1110      1120
EP0111 CTCTGCTCGCGTCAGAAGTTGAGCCGACCGTACACCGAGGTCACCATGATGACCCTGCTC
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
GSN:AA CTCTGCTCGCGTCAGAAGTTGAGCCGACCGTACACCGAGGTCACCATGATGACCCTGCTC
             1000      1010      1020      1030      1040      1050

        1130      1140      1150      1160      1170      1180
EP0111 ACCAGCATGGCAGACAAGGAGCTGGTCCACATGATCGCCTGGGCCAAGAAGCTCCCAGGT
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
GSN:AA ACCAGCATGGCAGACAAGGAGCTGGTCCACATGATCGCCTGGGCCAAGAAGCTCCCAGGT
             1060      1070      1080      1090      1100      1110

        1190      1200      1210      1220      1230      1240
EP0111 TTTCTGCAGCTGTCCCTGCACGATCAGGTGCTGCTGCTGGAGAGCTCGTGGCTGGAGGTG
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
GSN:AA TTTCTGCAGCTGTCCCTGCACGATCAGGTGCTGCTGCTGGAGAGCTCGTGGCTGGAGGTG
             1120      1130      1140      1150      1160      1170

        1250      1260      1270      1280      1290      1300
EP0111 CTCATGATCGGCCTCATTTGGAGGTCCATCCACTGTCCCGGGAAGCTCATCTTTGCACAA
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
GSN:AA CTCATGATCGGCCTCATTTGGAGGTCCATCCACTGTCCCGGGAAGCTCATCTTTGCACAA
             1180      1190      1200      1210      1220      1230
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```
       1310      1320      1330      1340      1350      1360
EP0111 GACCTCATCCTGGACAGGAATGAGGGAGACTGCGTGGAAGGCATGACGGAGATCTTCGAC
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
GSN:AA GACCTCATCCTGGACAGGAATGAGGGAGACTGCGTGGAAGGCATGACGGAGATCTTCGAC
             1240      1250      1260      1270      1280      1290

       1370      1380      1390      1400      1410      1420
EP0111 ATGCTGCTGGCCACTGCTTCCCGCTTCCGTGTGCTCAAACTCAAACCTGAGGAATTCGTC
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
GSN:AA ATGCTGCTGGCCACTGCTTCCCGCTTCCGTGTGCTCAAACTCAAACCTGAGGAATTCGTC
             1300      1310      1320      1330      1340      1350

       1430      1440      1450      1460      1470      1480
EP0111 TGCCTCAAAGCTATTATTTACTCAACTCCGGTGCTTTTTCTTTCTGCACCGGCACCATG
       :::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
GSN:AA TGCCTCAAAGCTATTATTTACTCAACTCCGGTGCTTTTTCTTTCTGCACCGGCACCATG
             1360      1370      1380      1390      1400      1410

       1490      1500      1510      1520      1530      1540
EP0111 GAGCCACTTCACAACAGCGCGGCGGTTCAGAGCATGCTGGACACCATCACAGACGCACTC
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
GSN:AA GAGCCACTTCACAACAGCGCGGCGGTTCAGAGCATGCTGGACACCATCACAGACGCACTC
             1420      1430      1440      1450      1460      1470

       1550      1560      1570      1580      1590      1600
EP0111 ATTCATTACATCAGTCAGTCGGGTTACTTGGCCCAGGAGCAGGCGAGACGGCAGGCCCAG
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
GSN:AA ATTCATTACATCAGTCAGTCGGGTTACTTGGCCCAGGAGCAGGCGAGACGGCAGGCCCAG
             1480      1490      1500      1510      1520      1530

       1610      1620      1630      1640      1650      1660
EP0111 CTGCTCCTGCTGCTCTCCCACATCAGGCACATGAGCAACAAAGGCATGGAGCACCTCTAC
       : ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
GSN:AA CCGCTCCTGCTGCTCTCCCACATCAGGCACATGAGCAACAAAGGCATGGAGCACCTCTAC
             1540      1550      1560      1570      1580      1590

       1670      1680      1690      1700      1710      1720
EP0111 AGCATGAAGTGCAAGAACAAAGTCCCTCTTTATGACCTCCTACTGGAGATGCTCGATGCC
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
GSN:AA AGCATGAAGTGCAAGAACAAAGTCCCTCTTTATGACCTCCTACTGGAGATGCTCGATGCC
             1600      1610      1620      1630      1640      1650

       1730      1740      1750      1760      1770      1780
EP0111 CACCGCCTGCACCACCCCGTCAGAGCACCCCAGTCCTTGTCCCAAGTCGACAGAGACCCT
       :::::::::::::::::::::::::: :::::::::::::::::::::::::::::::::
GSN:AA CACCGCCTGCACCACCCCGTCAGAGCCCCCCAGTCCTTGTCCCAAGTCGACAGAGACCCT
             1660      1670      1680      1690      1700      1710

       1790      1800      1810      1820      1830      1840
EP0111 CCCTCCACCAGCAGCGGCGGGGGTGGAATCGCTCCCGGTTCTATATCAGCATCTCGAGGC
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
GSN:AA CCCTCCACCAGCAGCGGCGGGGGTGGAATCGCTCCCGGTTCTATATCAGCATCTCGAGGC
             1720      1730      1740      1750      1760      1770

       1850      1860      1870      1880      1890      1900
EP0111 AGAATCGAGAGTCCGAGCAGAGGCCCCTTTGCTCCCAGTGTCCTTCAGTATGGAGGGTCG
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
GSN:AA AGAATCGAGAGTCCGAGCAGAGGCCCCTTTGCTCCCAGTGTCCTTCAGTATGGAGGGTCG
             1780      1790      1800      1810      1820      1830

       1910      1920      1930      1940      1950      1960
EP0111 CGTCCTGACTGCACCCCGGCCCTTCAAGACTGAGCACACAGTCCAAGGCCCTTTTTTTGT
       :::::::::::::::::::::::::::::::::
GSN:AA CGTCCTGACTGCACCCCGGCCCTTCAAGACTGA
             1840      1850      1860
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        1970      1980      1990      2000      2010      2020
EP0111 GGCTCAAGGGTTCAGGTTGGGACAAGGTGATGCTTGATTTAATTTTAAGAATTATTTATA
```

1 Yoll
translation of Seq ID 1
VS
Seq ID 3 of JP [illegible] 20168

```
ID   AAB20898 standard; Protein; 620 AA.
XX
AC   AAB20898;
XX
DT   05-JAN-2001  (first entry)
XX
DE   Oryzias lapites oestrogen receptor protein SEQ ID NO:3.
XX
KW   Oryzias lapites; oestrogen receptor.
XX
OS   Oryzias lapites.
XX
PN   JP2000201688-A.
XX
PD   25-JUL-2000.
XX
PF   06-APR-1999;    99JP-0098787.
XX
PR   10-NOV-1998;    98JP-0319465.
XX
PA   (SUMO ) SUMITOMO CHEM CO LTD.
XX
DR   WPI; 2000-567950/53.
DR   N-PSDB; AAA92175.
XX
PT   An estrogen receptor gene and its application
XX
PS   Claim 2; Page 13-15; 23pp; Japanese.
XX
CC   The present sequence represents an oestrogen receptor derived from
CC   Oryzias lapites. Also described are: (1) a vector comprising the
CC   oestrogen receptor gene; (2) a transformant prepared by introducing
CC   the oestrogen receptor gene or vector from (1) into a host cell;
CC   (3) a method for the preparation of an oestrogen receptor comprising
CC   culturing the transformant from (2) to produce the oestrogen receptor;
CC   and (4) a method for the evaluation of oestrogen receptor-activating
CC   ability of a chemical substance in which the chemical substance is
CC   reacted with a transformant prepared by introducing a reporter gene
CC   connected downstream of a transcription controlling region containing
CC   an oestrogen response sequence and the above oestrogen receptor gene to
CC   an oestrogen-nonendogenous host cell. The transformant can be used for
CC   the evaluation of oestrogen receptor-activating ability of a chemical
CC   substance.
XX
SQ   Sequence   620 AA;
SQ   39 A; 44 R; 10 N; 29 D; 0  B; 18 C; 27 Q; 31 E; 0  Z; 60 G; 19 H;
SQ   24 I; 67 L; 25 K; 20 M; 15 F; 43 P; 67 S; 30 T; 4  W; 20 Y; 28 V;
SQ   0 Others;
     mskrqssvqi rqlfgpalrs rispassele tlspprlspr dplgdmypee srgsggvaav
     dllegtydya apnpattply sqsstgyysa pletngppse gslqslgsgp tsplvfvpss
     prlspfmhpp shhylettst pvyrsshqga sredqcgsre dtcslgelga gagaggfema
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(75-1934:1-620)

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          1890      1920
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1 Y

This entry is from: SWALL (SPTR)

Save

Link

General Description References Comments Links Keywords Features Sequence

General information

Entry name	ESR1_ORYLA
Accession number	P50241
Created	Rel. 34, 1-OCT-1996
Sequence update	Rel. 37, 15-DEC-1998
Annotation update	Rel. 40, 16-OCT-2001

01-10-1996

1-5

5

Description and origin of the Protein

Description	ESTROGEN RECEPTOR (ER) (ESTRADIOL RECEPTOR) (ER-ALPHA).
Gene name(s)	ESR OR NR3A1 OR MER.
Organism source	Oryzias latipes (Medaka fish).
Taxonomy	Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi; Actinop Neopterygii; Teleostei; Euteleostei; Neoteleostei; Acanthomorpha; Acantho Percomorpha; Atherinomorpha; Beloniformes; Adrianichthyidae; Oryziinae;
NCBI TaxID	8090

References

[1] Okada,H., Kawahara,T., Yamashita,I., RL Submitted (APR-1998) to the EMBL/GenBank/DDBJ databases.

Position: RP SEQUENCE FROM N.A.

Comments: RC STRAIN=D-RR; TISSUE=LIVER;

[2] Kawahara,T., Yamashita,I.,
RT **"Oryzias latipes genomic DNA for estrogen receptor**.RL Submitted the EMBL/GenBank/DDBJ databases.

Position: RP SEQUENCE FROM N.A.

Comments

FUNCTION	THE STEROID HORMONES AND THEIR RI ARE INVOLVED IN THE REGULATION OF EUKARYOTIC GENE EXPRESSION AND A CELLULAR PROLIFERATION AND DIFFER IN TARGET TISSUES.
SUBUNIT	BINDS DNA AS A HOMODIMER. CAN FORI HETERODIMER WITH ER- BETA (BY SIMIL
SUBCELLULAR LOCATION	NUCLEAR.
DOMAIN	COMPOSED OF THREE DOMAINS: A MOD N-TERMINAL DOMAIN, A DNA-BINDING D(A C-TERMINAL STEROID-BINDING DOMAI
SIMILARITY	BELONGS TO THE NUCLEAR HORMONE I FAMILY. NR3 SUBFAMILY.

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Database cross-references

EMBL	D28954;BAA25900.1;-.
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InterPro	IPR000536;Hormone_rec_lig.
	IPR001292;Oest_recep.
	IPR001723;Strdhormone_receptor.
	IPR001628;zf-C4.
Pfam	PF00104;hormone_rec;1.
	PF02159;Oest_recep;1.
	PF00105;zf-C4;1.
PRINTS	PR00398;STRDHORMONER.
	PR00047;STROIDFINGER.
SMART	SM00430;HOLI;1.
	SM00399;ZnF_C4;1.
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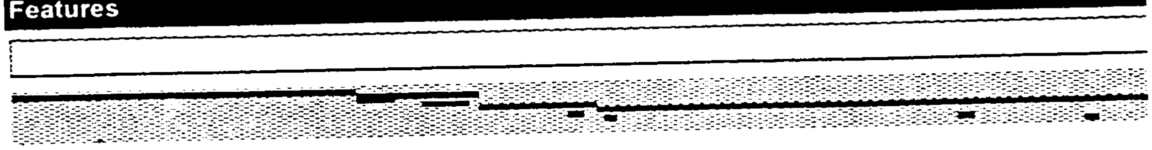
Keywords

Receptor; Transcription regulation; DNA-binding; Nuclear protein; Zinc-finger; Steroid-binding;

Features



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ZN_FING	222	246	25	C4-TYPE.
DOMAIN	252	314	63	HINGE.
DOMAIN	315	620	306	STEROID-BINDING.
DOMAIN	299	307	9	POLY-GLY.
DOMAIN	320	323	4	POLY-LEU.
DOMAIN	511	515	5	POLY-LEU.
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Sequence information

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General Description References Comments Links Keywords Features Sequence

SRS 6.1.3 | feedback

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overlap (75-1934:1-620)

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               810       840       870       900       930       960
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・先行技術文献

Winn R., Marine Environmental Research, vol.46(1-5), p.130 (1998)

Takagi S. et al., Molecular Marine Bilogy and Biotechnology, vol.3(4), pp.192-199 (1994)

Gray M.A. et al., Environmental Toxicology and Chemistry, vol.18(11), pp.2587-2594 (1999)

この先行技術文献調査結果の記録は、拒絶理由を構成するものではない。

ZOOLOGICAL SCIENCE **19**: 309–319 (2002)

Aryl Hydrocarbon Receptor is Required for Prevention of Blood Clotting and for the Development of Vasculature and Bone in the Embryos of Medaka Fish, *Oryzias latipes*

Toshiyuki Kawamura and Ichiro Yamashita*

Center for Gene Science, Hiroshima University, Kagamiyama 1-4-2, Higashi-Hiroshima 739-8527, Japan

ABSTRACT—The aryl hydrocarbon receptor (AHR) is a member of ligand-activated transcription factors and conserved among vertebrates. To investigate the role of AHR in fish development, medaka embryos were treated with agonist (2,3,7,8-tetrachlorodibenzo-*p*-dioxin), antagonists (α- naphthoflavone and resveratrol), and inhibitor (piperonyl butoxide) of cytochromes (Cyts) P450 encoded by a battery of target genes. These embryos were found to have similar abnormal phenotypes. Among the most consistent phenotypes were blood clotting and malformation of bone that were associated with vascular damages. These results thus indicate that control of AHR is important for proper development of fish embryos. AHR may control levels of Cyts P450 that are responsible for synthesis and metabolism of a toxic compound that caused the abnormal phenotypes. Complementary DNA fragments encoding AHR homologs were cloned from medaka embryos. AHR-specific mRNA was ubiquitously expressed in embryos and adult tissues.

Key words: aryl hydrocarbon receptor, blood clotting, bone formation, cytochrome P450, dioxin.

INTRODUCTION

Planar halogenated hydrocarbons, such as 2,3,7,8-tetrachlorodibenzo-*p*- dioxin (TCDD), are notorious environmental pollutants that are extremely toxic to early stages of vertebrate development (Peterson *et al.*, 1993). Hallmark signs of TCDD toxicity in fish sac fry are yolk sac edema, slowed blood flow, hemorrhage, and growth retardation culminating in mortality (Cantrell *et al.*, 1996; Henry *et al.*, 1997; Hornung *et al.*, 1999). Vascular damage, as assessed by TCDD-induced apoptotic cell death, is a key physiological mediator of the embryo toxicity (Cantrell *et al.*, 1996; Cantrell *et al.*, 1998). These chemicals bind to a ligand-dependent transcriptional factor called the aryl hydrocarbon receptor (AHR), resulting in the activation of a battery of genes encoding various cytochromes (Cyts) P450 that are responsible for degradation of the environmental contaminants (Hankinson, 1995; Guiney *et al.*, 1997; Guiney *et al*, 2000). AHR is conserved among vertebrates, thus, may have arisen in an ancestral vertebrate as a detoxification system.

Although, to date, an endogenous ligand for AHR has not been found, AHR is ubiquitously expressed in most

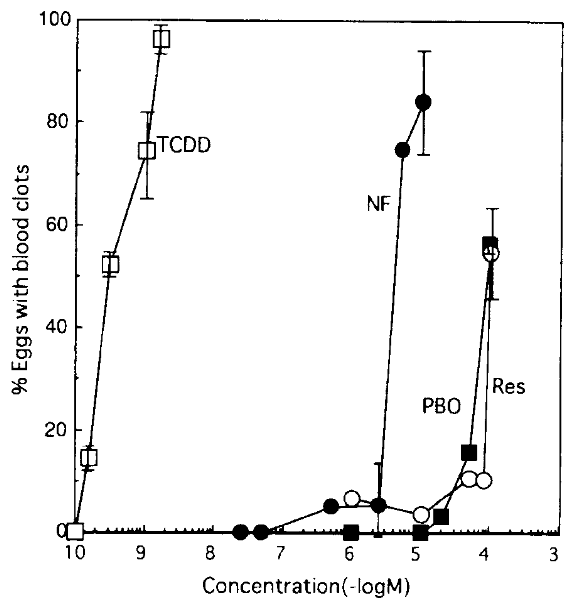

Fig. 1. Effects of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), α-naphthoflavone (NF), resveratrol (Res), and piperonyl butoxide (PBO) on blood clotting during the embryo stage. Eggs were treated with TCDD, NF, Res, or PBO at the indicated concentrations until 6, 6, 4, or 5 dpf, respectively, and counted for blood clots.

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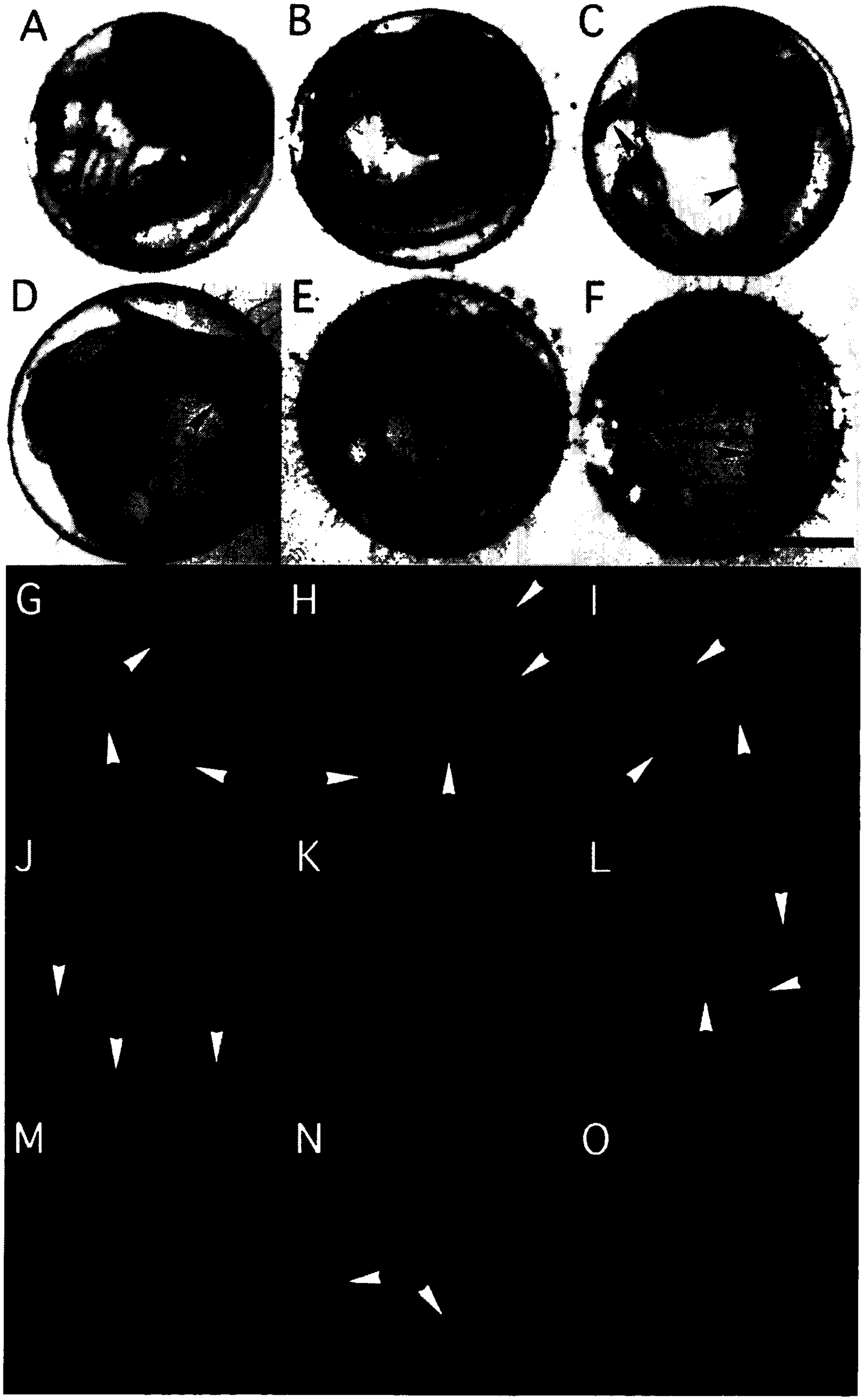
FAX. +81-824-22-7184.
E-mail: iyama@hiroshima-u.ac.jp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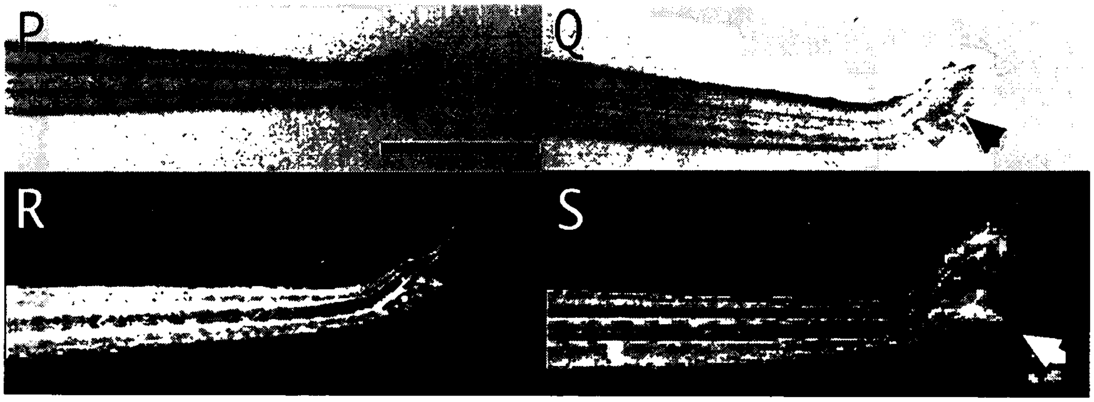

Fig. 2. Photographs of blood clots, yolk vein, and fin. Eggs and fry were treated with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), α-naphthoflavone (NF), resveratrol (Res), or piperonyl butoxide (PBO) as follows and photographed for blood clots (**A–F**), yolk vein under green fluorescence (**G–O**), and fin (**P–S**): (**A**) mock-treated, 5 dpf, (**B, C**) 1.55 nM TCDD, 5 and 7 dpf, (**D**) 10 μM NF, 5 dpf, (**E**) 100 μM PBO, 5 dpf; (**F**) 100 μM Res, 4 dpf, (**G–I**) mock-treated at 3, 5, and 7 dpf, (**J, K**) 1.55 nM TCDD, 5 and 7 dpf, (**L, M**) 10 μM NF, 3 and 5 dpf; (**N, O**) 100 μM PBO, 4 and 5 dpf, (**P, R**) mock-treated, 5-day post-hatching; and (**Q, S**) 0.155 nM TCDD, 5-day post-hatching. Arrows indicate blood clots (**B– F**, and **Q**), yolk veins (**G–J**, **L**, and **N**), and the constricted fin (**S**). Bar, 0.5 mm

organs and cells in the body (Rowlands and Gustafsson, 1997). However, there is only a limited knowledge of developmental and physiological functions of AHR in the mouse (Gonzalez and Fernandez-Salguero, 1998), although the role of AHR in detoxification of environmental aryl hydrocarbons has been extensively studied *in vitro* (Hankinson, 1995). AHR-null mice were resistant to the acute toxicity (Fernandez-Salguero *et al.*, 1996) of and the teratogenic response (Mimura *et al.*, 1997) to TCDD, and found to have a number of abnormal phenotypes such as decreased accumulation of lymphocytes in the spleen and lymph nodes and reduction in liver size that are associated with accelerated rates of apoptosis (Fernandez-Salguero *et al.*, 1995), and difficulties in reproduction (Abbott *et al.*, 1999; Robles *et al.*, 2000). Thus, AHR is involved in the toxicity of and the teratogenesis by TCDD *in vivo*, and plays an important role in the development of the liver and the immune system, and in reproduction. However, no such function has been elucidated in other vertebrates.

Here we re-evaluated the role of AHR in chemical toxicity of TCDD in medaka fish embryos because there have been no pharmacological studies in fish using antagonist and also examined for any possible developmental and physiological function of AHR in medaka fish embryos using antagonists and Cyts P450 inhibitor. We found that AHR mediates TCDD toxicity such as blood clotting, malformation of bone, and regression of blood vessels, and that AHR is required for the embryonic development of vasculature and bone. To our knowledge, this is the first report of the developmental role of AHR in lower vertebrates

MATERIALS AND METHODS

Fish and embryo culture

We used the d-rR strain of medaka fish, *O. latipes* (Kawahara and Yamashita, 2000) The fish were maintained at 25–26°C under artificial photo-period of 14L 10D, and fed by powdered Tetramin (Tetra) Eggs were collected within 12 hr postfertilization (hpf), rinsed with tap water, and immersed in Yamamoto's salt solution (Yamamoto, 1969) with or without test chemicals. At least 30 eggs were used in each experiment TCDD was purchased from Cambridge Isotope Laboratories, Inc Antagonists, α-naphthoflavone (NF)(Gasiewicz and Rucci, 1991; Merchant *et al.*, 1993) and resveratrol (Res)(Ciolino *et al.* 1998, Casper *et al.*, 1999; Singh *et al.*, 2000), were from Sigma. Cyts P450 inhibitor, piperonyl butoxide (PBO)(Dahl and Hodgson, 1979; Testa and Jenner, 1981; Adams *et al.* 1993), was from Tokyo Kasei Kogyo Co. These reagents were dissolved in acetone The stock solutions were diluted over 1,000-fold with Yamamoto's solution and added to eggs of 12 hpf for NF, Res and PBO or of 24 hpf for TCDD. The solvent was added to the mock-treated eggs as a control. The reducing agent, N-acetyl cysteine (NAC) (Sigma), was dissolved in Yamamoto's solution and added to 12 hpf eggs Eggs and fry were cultured under the same condition as above (except without feed) and inspected for blood clotting under a dissecting microscope. Eggs and fry in which blood clots formed were counted

Data are presented as mean±SEM Statistical significance between values of control and experiment was assessed by Student's *t*-test

Observation of blood vessels

In order to observe the development of blood vessels, eggs were fixed with 4% paraformaldehyde for 3 days and observed under green fluorescence with a filter set (excitation filter, 546/10 nm, barrier filter, 590 nm) in Leica MZ FLIII stereo-fluorescence microscope The fixed eggs were also dechorionated with forceps and stained with hematoxylin

Bone staining

In order to observe the bone development, calcified bone was stained with alizarin S essentially as described (Takeuchi, 1960). In brief, fish were anesthetized with 0.015% phenylurethane, skinned with forceps, treated with 2% KOH for 24 hr, and finally stained with 0.1% alizarin S solution After washing in tap water, the fish were successively transferred to 50% and 70%, and finally embedded in 100% glycerin Anesthetized fry were directly treated with 2% KOH for 2 h, fixed in 4% paraformaldehyde for 24 hr, then stained with alizarin S.

Isolation of cDNAs encoding medaka AHR homologs

As PAS domain of AHR is highly conserved among vertebrates (Rowlands and Gustafsson, 1997), a corresponding region of cDNA was amplified with degenerated oligonucleotides (AhR-A1 and AhR-B1) as described (Hahn and Karchner, 1995) using total RNA from 6-day postfertilization (dpf) medaka embryos. The cDNA fragment was cloned in plasmid and sequenced. Based on the sequence, nested oligonucleotides were designed and 5' and 3' RACEs (rapid amplification of cDNA ends) were performed on the same RNA by using 5' and 3' RACE Systems (GIBCO BRL), yielding the remainder of the coding sequence, 5' and 3' untranslated regions, and polyadenylation sequence.

RNA analysis

Total RNA was extracted from embryos and adult tissues as described (Kawahara *et al.*, 2000). RT-PCR (reverse transcription-polymerase chain reaction) analysis was done as described (Kawahara *et al.*, 2000) with the primers as follows for generation of the 437-bp cDNA encoding a part of PAS domain: poly(dT) oligonucleotide used for RT, and 5'-CCAGCAGGAGTTCAGGAGGA and 5'-ATTTTACCCTTTGCGTCACA for PCR. Amplified DNA was electrophoresed in 1% agarose gel and stained with ethidium bromide.

RESULTS

AHR mediates the toxic effects of TCDD on vascular development

We re-evaluated the toxic effects of TCDD on medaka embryos. To do this, embryos (1 dpf) were immersed in saline solution for medaka containing increasing concentrations of TCDD, and observed for any abnormal phenotype under a dissecting microscope (Fig. 1). Clearly visible signs of blood clotting were apparent after 4 days in caudal veins of TCDD-treated embryos (Fig. 2B), although blood cells were circulating in vasculature (Fig. 2J) but at a reduced rate. Blood clots were also found in yolk veins after 6 days (Fig. 2C), at that time, vascular structure was almost absent (Fig. 2K). In control embryos, yolk veins were apparent at 3 dpf (Fig. 2G) and developed progressively in a curve structure (Fig. 2A, H and I). Very small blood clots were occasionally found in yolk veins of normal embryos (less than 3%), but not scored in this study. These results are consistent with the previous observation that TCDD induces apoptosis of blood vessels (Cantrell *et al.*, 1996).

If TCDD induced the vascular damage through activation of AHR, the antagonist (NF) would reduce the extent to which blood clotting was detected. For this purpose, two different experiments were done, in which embryos were treated with high (1.55 nM) or medial (0.775 nM) concentration of TCDD (Fig. 3A or B, respectively). For both cases, addition of NF effectively suppressed blood clotting but only transiently (Fig. 3A and B). However, in the latter case, NF markedly enhanced the hatching success of TCDD-treated embryos, giving rise to almost complete hatching (Fig. 3C). These results indicate that TCDD-induced vascular damage is mediated through activation of AHR.

It is well known that TCDD-bound AHR activates transcription of a battery of genes encoding Cyts P450. If these

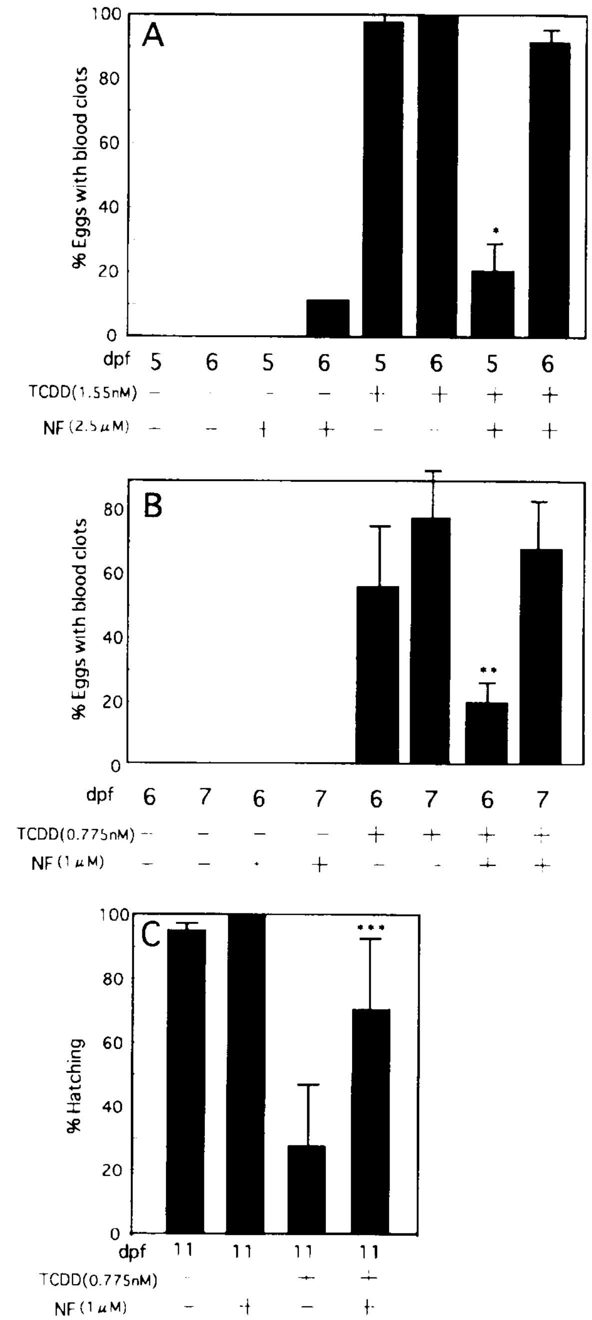

Fig. 3. Suppression by α-naphthoflavone (NF) of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD)-induced blood clotting and mortality. (**A**) Eggs were treated with 1.55 nM TCDD and 2.5 μM NF until 5 and 6 dpf as indicated, and examined for blood clotting. $^{*}P<0.01$. (**B**) Eggs were treated with 0.775 nM TCDD and 1 μM NF until 6 and 7 dpf as indicated, and examined for blood clotting. $^{**}P<0.2$. (**C**) Eggs were treated as described in (**B**), and examined for hatching rate at 11 dpf. $^{***}P<0.05$.

enzymes were involved in the TCDD-induced toxicity, an inhibitor of P450 would reduce the rate of TCDD-induced blood clotting. We therefore examined the ability of PBO to provide protection against high concentration (1.55 nM) of

TCDD (Fig. 4A). Unexpectedly, PBO reduced the blood clotting rate only slightly; we cannot use higher concentrations of PBO because PBO itself induced blood clotting (described below). We therefore tried to seek for conditions under which lower concentrations of TCDD induce blood clotting effectively. We found that blood clots formed in the caudal fin (Fig. 2Q) after immersing embryos until 5-day post-hutching at subnanomolar concentrations of TCDD (Fig. 4B). Blood clots did not form in the control fin (Fig. 2P). Under the above condition, PBO effectively suppressed the adverse effect of TCDD (Fig. 4C). These results suggest that the TCDD-induced toxicity was caused by elevated expression of a certain Cyt P450.

Previous reports conclude that oxidative stress caused by TCDD-induced expression of Cyts P450 contributes to embryotoxicity and vascular damage associated with apoptosis, because the reducing agent, NAC, partially recovers

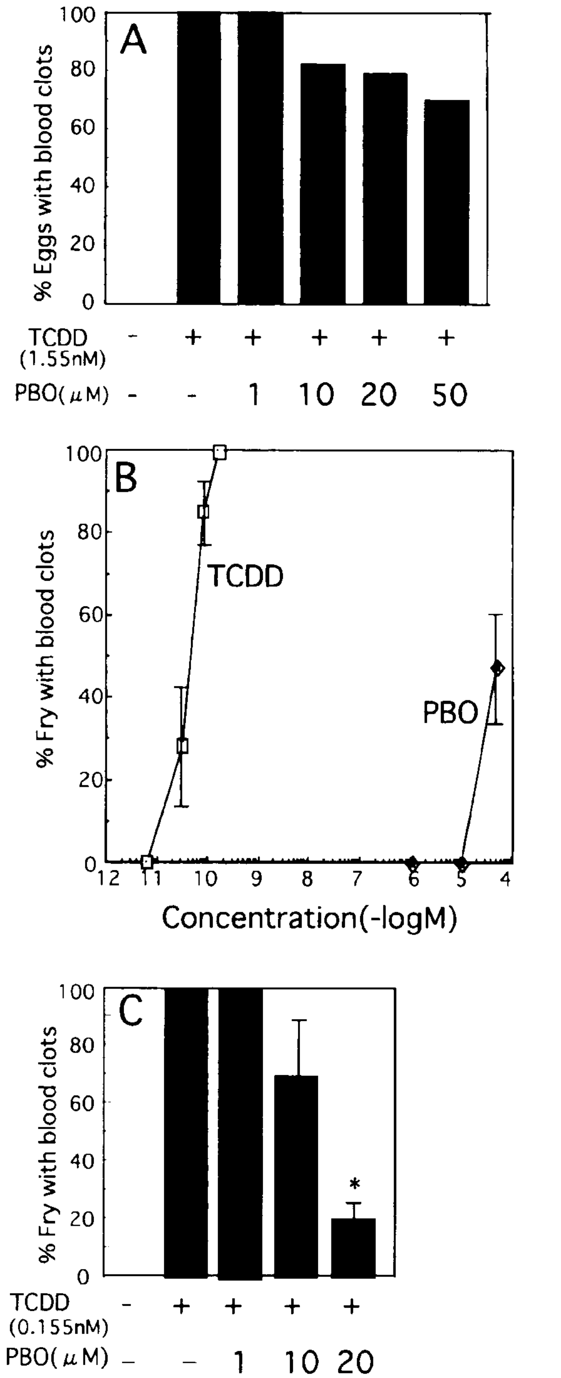

Fig. 4. Suppression by piperonyl butoxide (PBO) of 2,3,7,8- tetrachlorodibenzo-*p*-dioxin (TCDD)-induced blood clotting. (**A**) Eggs were treated with 1.55 nM TCDD and increasing concentrations (μM) of PBO as indicated until 5 dpf, and examined for blood clotting. (**B**) Eggs were treated with TCDD and PBO at the indicated concentrations until 5-day post-hatching, and examined for blood clotting in the caudal fin. (**C**) Eggs were treated with 0.155 nM TCDD and increasing concentrations of PBO as indicated until 5-day post-hatching, and examined for blood clotting in the caudal fin. $^{*}P<0.05$.

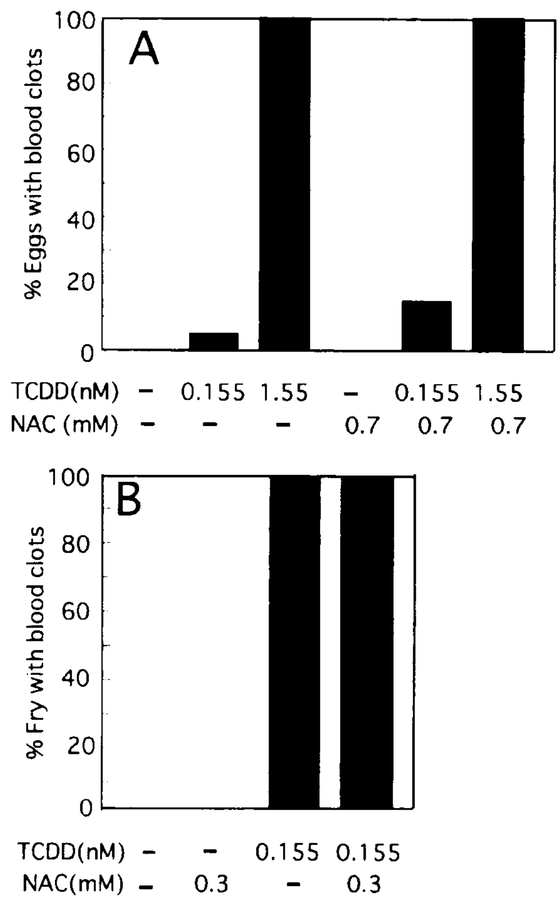

Fig. 5. N-acetyl cysteine (NAC) fails to suppress the 2,3,7,8- tetrachlorodibenzo-*p*-dioxin (TCDD)-induced blood clotting. (**A**) Eggs were treated with TCDD (nM) and NAC (mM) at the indicated concentrations until 5 dpf, and examined for blood clotting. (**B**) Eggs were treated with 0.155 nM TCDD and 0.3 mM NAC as indicated until 5-day post-hatching, and examined for blood clotting in the caudal fin.

the TCDD-induced embryotoxicity (Cantrell *et al.*, 1996): they observed 41% survival of the embryos that had been treated with 28 nM TCDD for 2 hr and released in 0.1 mM NAC until 3 days posthatch, in contrast to 2% survival of the embryos that had been treated with TCDD and released in water. The ability of NAC to inhibit TCDD-induced toxicity was re-assessed by adding 0.7 mM (Fig. 5A) or 0.3 mM (Fig. 5B) NAC to eggs before and during the treatment with TCDD. NAC could not inhibit the blood clotting induced by 0.155 or 1.55 nM TCDD. NAC itself induced blood clotting at more than 0.9 mM (data not shown). These results suggest that general oxidative stress is not responsible for the TCDD-induced blood clotting.

Vascular damage induced by antagonists (NF and Res) and Cyts P450 inhibitor (PBO)

At the initial experiments determining the concentrations of reagents used, we found that NF, Res, and PBO induced blood clotting at higher concentrations than those used for suppression of TCDD-induced toxicity (Fig. 1). Blood clots formed in caudal and yolk veins (Fig. 2D-F). Yolk veins developed normally at the early time of incubation (up to 4 dpf) (Fig. 2L and N), but their regression was apparent at the time when blood clots formed in yolk veins (at 5 dpf) (Fig. 2M and O). These results suggest that either inactivation of AHR by NF and Res or inhibition of certain Cyts P450 by PBO caused vascular damage and blood clotting.

If the hypothesis were true, antagonist of AHR and Cyts P450 inhibitor would act synergistically to cause toxicity. We examined the synergy between low concentrations of NF (2.5 μ M) and PBO (20 μM) that alone did not show any effect. Combination of these chemicals clearly increased the rate of blood clotting (Fig. 6). We therefore conclude that control of AHR activity and levels of Cyts P450 is required for proper development of vasculature in fish.

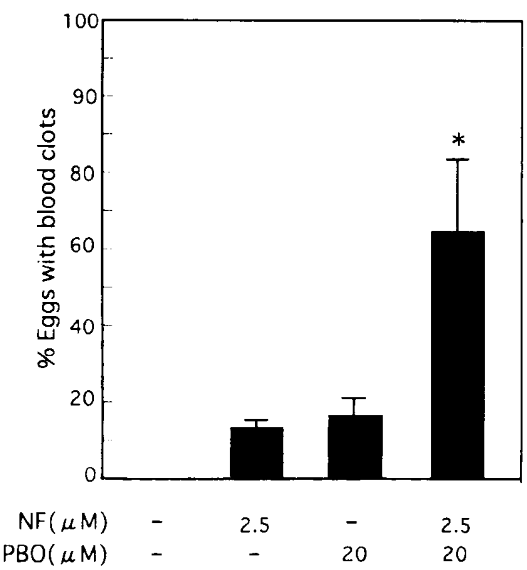

Fig. 6. Synergistic effects of α-naphthoflavone (NF) and piperonyl butoxide (PBO) on blood clotting. Eggs were treated with NF and PBO at the indicated concentrations (μM) until 6 dpf, and examined for blood clotting. $^{*}P<0.2$.

Malformation or degeneration of bone induced by TCDD, NF, and PBO

During the experiments by incubating eggs with lower concentrations of TCDD (less than 80 pM) until 7 dpf, most eggs developed normally in appearance and blood clots did not form. The eggs were transferred to Yamamoto's solution, then to aquaria after hatching, and reared to adult by normal diet as usual. Unexpectedly we found that these fish were deformed in shape like wavy mutants (Takeuchi, 1960). We examined the bone development by staining with alizarin S. The vertebral column of TCDD-treated fish curved dorso-ventrally and laterally (Fig. 7A and B). Neural and haemal spines were short in length and deformed (Fig. 7B). NF also suppressed the TCDD-induced toxicity on bone formation (Fig. 7C), indicating the involvement of AHR.

We examined the effect of TCDD on the embryonic bone formation by incubating eggs with TCDD until 5 days post-hatching. The staining of the fry with alizarin revealed the absence of calcification in the posterior region of spinal cord and in spines (Fig. 7D and E). We also found that caudal fins were round in shape and constricted (indicated by arrow in Fig. 2S) in the TCDD-treated fry.

In order to examine the possible function of AHR and Cyts P450 in the embryonic bone formation, eggs were treated with NF or PBO until 5 days post-hatching. The treatment with NF (2.5 μM) did not cause blood clotting in any portion of the fry (data not shown), which was different from the result with TCDD (Figs. 2Q and 4B). However, the treatment also caused degeneration of the posterior end of the spinal cord, but with normal development of spines (Fig. 7D, data not shown). PBO (50 μM) also caused the same defect in bone formation as that NF did (data not shown).

We further examined whether NF affects homeostasis of adult fish. To do this, adult fish which had been reared by normal diet for 2 months were fed by NF-containing diet (2 mg NF/g diet) for 2 months. During the cultivation, population of fish lacking posterior fins including anal, caudal, and dorsal fins appeared after a month and became increasing near to 100% by two months (Fig. 7F).

Taken together, these results suggest that hyperactivation of AHR by TCDD is toxic to the embryonic development of bone and caudal fin, that AHR is required for proper development of bone and homeostasis of posterior fins, and that a certain Cyt P450 is also required for bone development.

Isolation and characterization of cDNAs encoding AHR homologs of medaka fish, and ubiquitous expression of AHR mRNA

We first obtained four independent cDNA clones (clones 1, 2, 3 and 4) corresponding to PAS domain (Fig. 8A). These clones were found to be identical by sequencing.

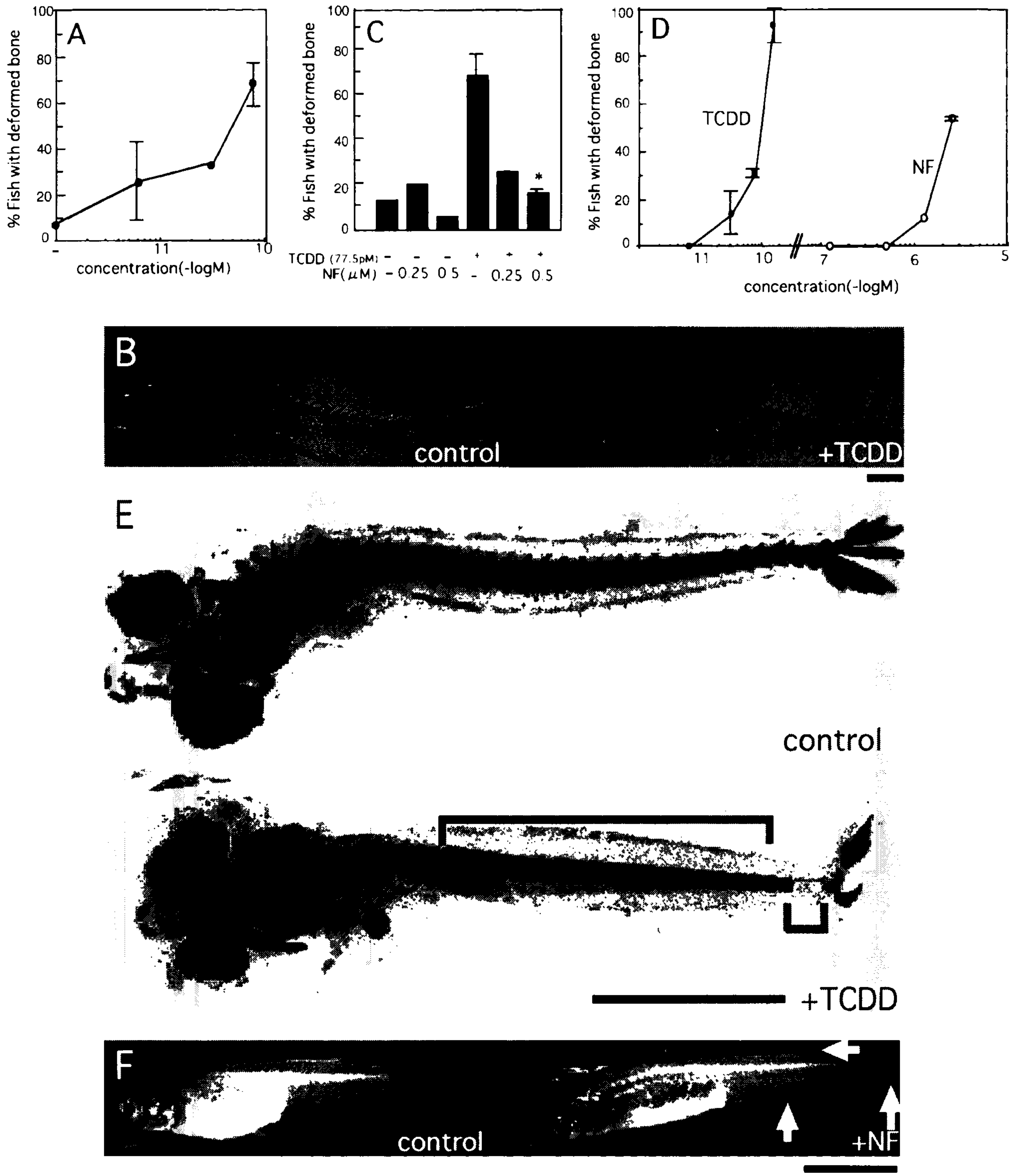

Fig. 7. Effects of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and α- naphthoflavone (NF) on bone formation. (**A**) Eggs were treated with TCDD at the indicated concentrations until 7 dpf, and reared to adult under TCDD-free condition. The adult fish were examined for bone formation after staining with alizarin. (**B**) Alizarin-stained bone of mock-treated (control) and TCDD (77.5 pM)-treated fish in (**A**). (**C**) Eggs were treated with 77.5 pM TCDD and NF at the indicated concentrations (μM) until 7 dpf, reared to adult under normal condition, and examined for bone formation. *P<0.05. (**D**) Eggs were treated with increasing concentrations of TCDD and NF until 5-day post-hatching, and examined for bone formation. (**E**) Alizarin-stained bone of mock- (control) and TCDD (0.155 nM)-treated fish in (**D**). Spines and posterior spinal bone are absent in the TCDD-treated fry as noted. (**F**) Normal adult fish were fed by NF-containing diet (2 mg NF/g diet) for 2 months, and photographed. Arrows indicate the degenerated fins. Bar, 1 mm in (**B**) and (**E**), and 5 mm in (**F**).

Next, 5' and 3' RACEs were performed, yielding four (clones 15, 24, 27 and 30) and six (clones 307–309, 314, 315, and 319) independent clones, respectively (Fig. 8A). Four clones from 5' RACE were identical. Six clones from 3' RACE were subdivided into three identical pairs, which differ from each other only in the 3' proximal sequences denoted by broken and dotted lines in Fig. 8A. Thus, we obtained three different cDNAs, named ahr-1, -2, and -3 (DDBJ accession numbers AB065092, AB065093, and AB065094, respectively). However, ahr-1 and ahr-3 encoded the same protein (AHR1α), and ahr-2 encoded another homolog (AHR1β). AHR1α and AHR1β differ from each other in the C-terminal peptides (amino acid 780–879 and 780–784) denoted by shaded and dotted boxes (Fig. 8A).

AHR1α and AHR1β are composed of 879 and 784 amino acids with calculated molecular weights of 95.5 and 85.3 KDa, respectively. Both proteins may be classified into a type of AHR1 because they are most homologous to AHR1 of the teleost *Fundulus heteroclitus* (Karchner *et al.*, 1999) (Fig. 8B). The medaka AHR1α and AHR1β are also composed of three conserved domains such as basic-helix-loop-helix (bHLH), Per-ARNT-Sim (PAS), and glutamine-rich

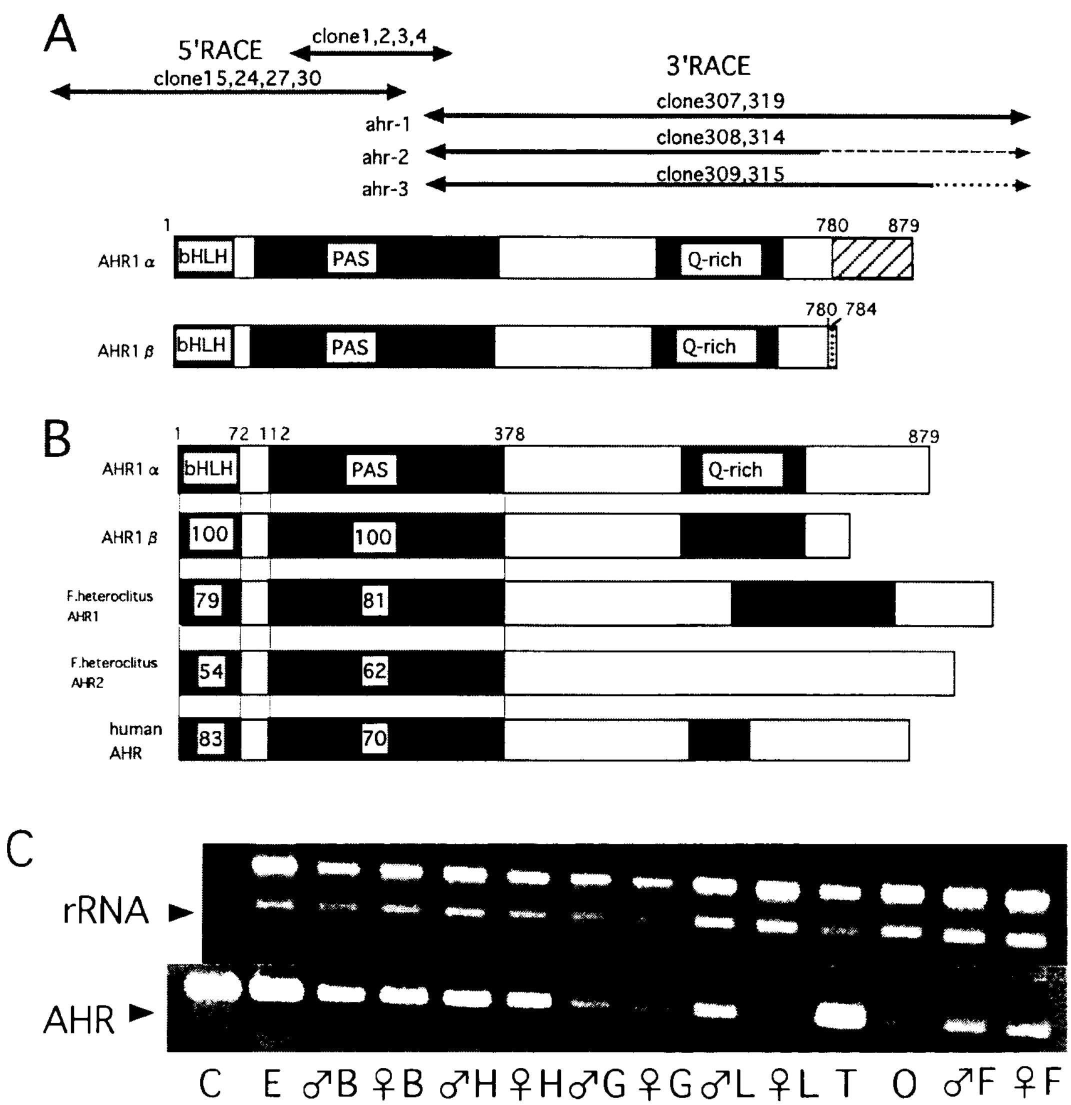

Fig. 8. Schematic drawing of the cDNAs cloned and the deduced proteins, and ubiquitous expression of AHR mRNA. (**A**) Inserts in the plasmid clones are shown on the deduced proteins (AHR1α and AHR1 β). Plasmid numbers are marked on the corresponding inserts. The three cDNAs which differ from each other only in the 3' terminal sequences (denoted by broken and dotted lines) are named ahr-1, -2, and -3. AHR1α and AHR1β, in which three conserved domains are marked by bHLH, PAS, and Q-rich, differ from each other only in the C-terminal short peptides marked by shaded and dotted boxes. (**B**) Identity (%) of amino acid sequence among bHLH and PAS domains of AHRs from medaka, *F. heteroclitus* (killifish), and human (Dolwick *et al.*, 1993). (**C**) RT-PCR analysis of total RNAs from medaka embryos (6 dpf) and adult tissues. Symbols: B, brain; C, the control band amplified from the cDNA; E, embryo; F, caudal fin; G, gill; H, heart; L, liver; O, ovary; and T, testis. Ribosomal RNAs in the RNA samples are also shown.

(Q) domains (Rowlands and Gustafsson, 1997) (Fig. 8B).

Expression of AHR mRNA was analyzed by RT-PCR on total RNAs prepared from medaka embryos and adult tissues such as brain, fin, gill, heart, liver, ovary, and testis. AHR mRNA was detected in all samples tested, and in large amounts in embryos and testis (Fig. 8C).

DISCUSSION

TCDD-induced vascular and bone damages through hyperactivation of AHR

TCDD is the most potent toxicant for vertebrate species. Exposure of vertebrate embryos to TCDD can result in various acute and chronic toxicities such as reproductive failure, teratogenic abnormalities, and immunological dysfunction (Peterson *et al.*, 1993). In fish, vascular damage is the most pronounced adverse effects of TCDD exposure during embryonic development. Vascular hemorrhaging, regression of blood vessels, pericardial sac edema, and reduced circulation are hallmark indicators that vascular function is compromised in the developing embryos (Cantrell *et al.*, 1996; Henry *et al.*, 1997; Hornung *et al.*, 1999; Guiney *et al.*, 2000). The vascular lesions have been demonstrated to be associated with apoptosis and induced expression of Cyt P450 1A in blood vessels of medaka embryos (Cantrell *et al.*, 1998). In the present study, we reexamined the TCDD-induced vascular damage in medaka embryos by observing blood clotting and regression of blood vessels. We found that these vascular damages can be suppressed, but transiently, by antagonist, NF (Fig. 3), giving a convincing evidence that the TCDD-induced vascular damage is mediated through hyperactivation of AHR. The transient suppression may be explained by the fact that TCDD, but not NF, is very stable *in vivo* against catabolic activities of Cyts P450 (Miniero *et al.*, 2001). Although the damage can also be suppressed by Cyts P450 inhibitor, PBO (Fig. 4C), general oxidative stress caused by Cyts P450-mediated oxidative reactions may not be responsible for the TCDD-induced damage, in inconsistent with the previous conclusion (Cantrell *et al.*, 1996), because reducing agent, NAC, could not recover the damage in vasculature (Fig. 5) or also in bone (data not shown). We assume that a toxic compound that may be accumulated *in vivo* by elevated levels of Cyt P450 is responsible for the TCDD-induced pathology (Fig. 9).

We also found that embryonic treatment with picomolar concentrations of TCDD causes malformation of bone in adult fish (Fig. 7). The treatment did not give apparent complications including blood clotting in the hatching fry, thus, the bone staining is the most sensitive method for detecting TCDD toxicity. The bone deformity could also be recovered by co-treatment with the antagonist (Fig. 7C), implying the role of hyperactivated AHR. TCDD may directly act on bone, because it inhibits osteogenesis in bone-forming cultures of chicken and rat cells (Gierthy *et al.*, 1994; Singh *et al.*, 2000). Treatment of medaka fish with TCDD from the egg stage to post-hatching also caused developmental defects in bone formation at the posterior region of vertebral column and at spines (Figs. 7D, E). However, it may be possible that these defects occurred secondarily to vascular damage, because blood clots formed at the base of the caudal fin under the same condition (Fig. 2Q).

AHR is required for prevention of blood clotting and for proper development of vasculature and bone in medaka fish

AHR is conserved among vertebrates, and ubiquitously expressed in embryos and adult tissues. In the present study, we have cloned three different cDNAs encoding two AHR homologs from medaka fish, O. latipes (Fig. 8). The two homologs obtained may belong to a type of AHR1 by amino acid sequence similarity, thus named AHR1α and AHR1β. They differ from each other only in C-terminal short peptide, and may be derived from alternative splicing. AHR1 mRNA was also ubiquitously expressed in medaka embryos and adult tissues, suggesting developmental and physiolog-

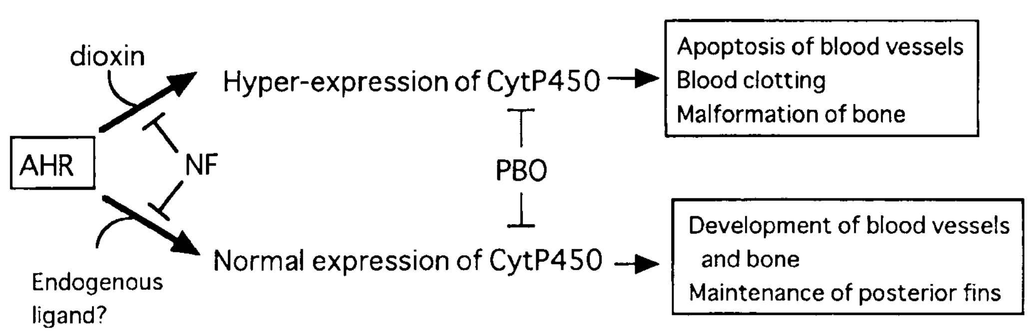

Fig. 9. Model for the role of AHR in the TCDD (dioxin)-induced toxicity, the development of blood vessels and bone, and the maintenance of posterior fins in the medaka fish, *O. latipes*. TCDD-bound AHR induces hyper-expression of a certain Cyt P450, resulting in the toxicities such as apoptosis of blood vessels, blood clotting, and malformation of bone. Either the antagonist (NF) or the Cyts P450 inhibitor (PBO) can suppress the TCDD-induced toxicity. An endogenous ligand is bound to and constitutively activates AHR. The activated AHR is responsible for normal expression of a certain Cyt P450 that is required for the development of blood vessels and bone and homeostasis of posterior fins. *In vivo* inhibition of AHR and Cyt P450 by NF and PBO, respectively, causes developmental abnormalities in vasculature and bone.

ical roles in medaka fish.

To investigate the role of AHR in fish development and physiological homeostasis, medaka embryos (12 hpf) were treated with the antagonists, NF and Res. These compounds did not cause any apparent defects until 4 dpf, but displayed developmental toxicities such as blood clotting and regression of blood vessels at 5 dpf (Figs. 1 and 2). Blood clotting may be caused by regression of blood vessels, because platelet adhesion to subendothelial collagens and activation by components of the extracellular matrix are crucial for blood coagulation (Nieswandt *et al.*, 2001). NF also caused the malformation of bone at 5-day post-hatching (Fig. 7D) and the regression of posterior fins such as anal, caudal, and dorsal fins at the adult period (Fig. 7F). These results suggest the presence of an endogenous ligand for AHR and that constitutive activation of AHR is specifically required for the development of blood vessels and bone and for the maintenance of posterior fins (Fig. 9).

Ligand-bound AHR activates transcription of a battery of genes including various Cyts P450. If levels of a certain Cyt P450 were controlled by AHR bound to an endogenous ligand and required for proper development of blood vessels and bone, the well-known inhibitor (PBO) of the enzymatic activity of Cyts P450 would induce the same developmental defect as did the antagonist. Treatment of embryos with PBO specifically induced blood clotting, regression of blood vessels (Figs. 1 and 2), and degeneration of the posterior end of spinal cord (data not shown) at the same developmental stage as did the antagonist, suggesting the importance of Cyt P450, the identity of which is, however, unknown (Fig. 9). The synergistic effects exerted by NF and PBO (Fig. 6) also support the hypothesis. We assume that a certain Cyt P450 is responsible for degradation (or catabolism) of a toxic compound that caused the developmental abnormalities.

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Systematics and Zoogeography

The killifishes, or Cyprinodontiforms are small fresh and brackish water fishes of worldwide distribution in tropical and temperate latitudes.

Previous classfication of the order Cyprinodontes

The classification of the order Cyprinodontes Agassiz (equivalent to Microcyprini Regan) has been worked out by Gill (1865, 1874), Regan (1909, 1911), Hubbs (1924, 1926) and Myers (1931, 1938). The classification followed here is mostly according to Hubbs and Myers and is cited from Kulkalni (1940) who erected a new family Horaichthyidae represented by a remarkable Indian henpecked killifish, *Horaichthys setnai.Ê* However, substituting for the terms Amblyopsoidea and Poecilioidea, the suborders Amblyopsoidei and Cyprinodontoidei are used here, respectively. The subfamily Tomeurinae is removed from the family Poeciliidae to erect a new family Tomeuridae as suggested by Hubbs in his letter to S. L. Hora (India) in 1938. Representative genera are given in parentheses following family names.

Order Cyprinodontes (Microcyprini)

Suborder Amblyopsoidei

Family Amblyopsidae *(Chologaster, Amblyopsis)*

Suborder Cyprinodontoidei

Family Cyprinodontidae *(Cyprinodon, Fundulus,*

Aplocheilus, Panchax, Oryzias)

Family Goodeidae *(Goodea)*

Family Poeciliidae *(Poecilia, Gambusia, Xiphophorus)*

Family Jenynsiidae *(Jenynsia)*

Family Anablepidae *(Anableps)*

Family Tomeuridae *(Tomeurus)*

Family Adrianichthyidae *(Adrianichthys, Xenopoecilus)*

Family Phallostethidae *(Phallostethus, Gulaphallus)*

Family Horaichthyidae *(Horaichthys)*

The classification listed here has been generally held by ichthyologists until 1962.

As to the status of *Oryzias*, Myers (1931) considered it to represent a monogeneric tribe of the subfamilily Fundulinae. Later (1956), he revised his earlier classification, and considered *Oryzias* to represent a monogeneric subfamily of the Cyprinodontidae, the Oryziatinae.

Classification of new order Atherinoformes

Rosen (1962) presented evidence which indicates a relationship of the Amblyopsidae (North American cave fishes) with the percopsiform genera and, more distantly with the gadiforms. He isolated the cave fishes as a new order, the Amblyopsiformes, and recommended its alignment near the Percopsiformes and Gadiformes in a phyletic sequence.

In 1964, Rosen has made drastic taxonomic re-arrangements of the halfbeaks, killifishes, silversides, and their relatives. The outset of his re- arrangements was osteological analyses of the adrianichyid fishes of Celebes, which were found to have a mixture of beloniform, cyprinodontiform, and mugilform features. Then, his investigation was broadened to include representatives of all these groups as well as a species of phallostethid.

In consequence of reasonable osteological diagnoses, he erected a new order Atherinoformes which includes the excoetoids, scomberesocoids. On the basis of osteological evidence, he separated the medaka (*Oryzias*) from cyprinodontoids, placed it in adrianichthyoids and erected a new family Oryziatidae.

To visualize Rosen's account on osteological difference between cyprinodontoids and adrianichthyoids, the presentation of the schema of the skull of the generalized teleosts as shown in Fig. 2-1 may be apropos.

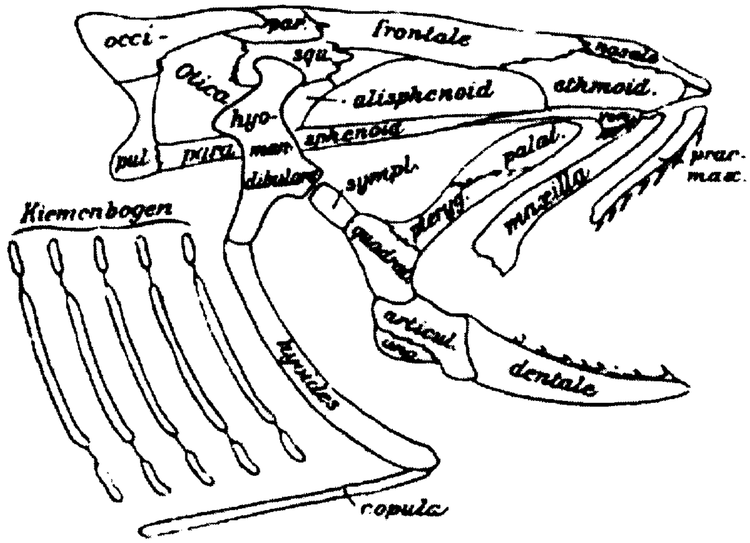

Fig. 2-1. A diagam of teleostean skull. Opercula and Infraorbitalia are removed. ang._angular, articul.= articular, occi. = occipital, palat. = palatine, p = quadrate, squ.= squamosa, sympl. = symplectic, vom. = vomer.

After R. Goldschmidt' E. Selenkas Zoologishes Taschenbuch fur Studierende. 1912 Leipzig, George Thieme.

In cyprinodontoid killifishes, bones of the jaws and the palatoquadrate arch are in such a construction that the premaxilla is protractile. In adrianichthyoid killifishes, on the other hand, the premaxillae are not protractile. The Adrianichthyidae are fishes of small size confined to the fresh-water lakes of Celebes. Two species, *Adrianichthys kruyti* and *Xenopoecilus sarasinorum,* are known. *Xenopoecilus* is characterized by having a large horse-shoe shaped mouth, an enormous ethmoideum and a single, median supraoccipital process formed by fusion of embryologically paired elements; "a cup-like excavation on the distal tip of the autopalatine that is capped by a large ball of cartilage and a discoidal sesamoid bone; a dorsal enlargement of the palatopterygoid arch with a prefrontal (Fig. 2-2); a maxilla that is carried on the upper edge rather than on the outer face of the posterior end of the premaxilla; a premaxilla that lacks a hooked or pointed posteroventral process; a tremendously reduced articular bone without a coronoid process that is almost wholly contained within the posterior part of the dentary; the articulation of the first pleural rib on the third rather than on the second vertebra; pelvic girdles that are not in contact medially and that have a long lateral spur extending upward between ribs; a dorsoventrally asymmetrical caudal skeleton with one or two very slender, rod-like epurals, and a caudal fin that is divided into indistinct upper and lower lobes by having a large gap between rays that articulate with the upper and lower hypural plates on the terminal half-centrum. (Rosen, 1964)

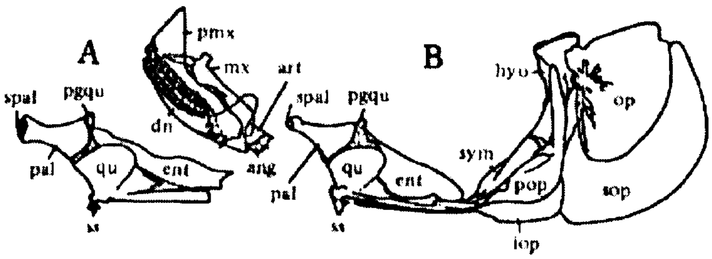

Fig. 2-2. Jaws and jaw suspension in adrianichthyoid killifishes. A. Jaws and palatopterygoid arch in *Oryzias latipes* (Temminck and Schlegel). b. Jaw suspension and opercular apparatus in *Xenopoecillus sarasinorum* (Popta). Note sesamoid bone below quadrate and bony cap over tip of palatine in A. and B. Note in A that lower arm of premaxilla lies over maxilla, large coronoid process on dentary, and absence of similar coronoid elevation on articular. Ang = angular, art = articular, dn = dentary, ent = entpterygoid (mesopterygoid), hyo = hyomandibular, iop = interoperculum, mx = maxilla, op = operculum, pal = palatine (autopalatine with or without dermopalatine), pgqu = pterygoquadrate cartilage, pmx = sesamoid bone capping autopalatine, ss = sesamoid bone, sym =symplectic. Rosen, 1964.

Rosen pointed out that except for the enlarged jaws and the presence of a median supraoccipital process, all the above features described within quotation marks can be identified in *Oryzias* (Fig. 2-2) but in no other killifishes so far as known.

It is therefore apparent that adrianichthyids and the medaka are intimately related and that they constitute a distinct subgroup of the killifishes, the adrianichthyoids, containing the families Adrianichthyidae *(Adrianichthys and Xenopoecilus),* Oryziatidae *(Oryzias),* and Horaichthyidae *(Horaichthys),* in contrast to the remainder of the families which are grouped together as cyprinodontoids (Cyprinodontoidea). Basing on Rosen's (1964) findings, Turner (1965) conveniently enumerated difference between cyprinodontoids and *Oryzias* as follows:

	Cyprinodontoidae	*Oryzias*
1.	First pleural rib on second vertebra.	First pleural rib on third vertebra.
2.	Pelvic girdle bones joined mid-ventrally; no upright lateral spur.	Pelvic girdle bones not joined mid-ventrally; an upright lateral spur present.
3.	Lower end of premaxilla bone expanded or hooked and sandwiched between the lower end of maxilla bone and dentary bone (lower jaw).	Lower end of premaxilla bone not expanded, and dorsal to the maxilla bone rather than between it and the dentary bone.
4.	Hypural plates often fused.	Hypural plates never fused.
5.	Hypochordal musculature entirely absent.	Hypochordal musculature present.
6.	Caudal fin never incipiently lobed.	Caudal fin incipiently lobed.

The family Horaichthyidae erected by Kulkarni (1940) comprises a single species, *Horaichthys setnai.* It is a small translucent oviparous fish inhabiting brackish waters and estuaries in the province of Bombay, India. Osteological study (Kulkalni 1948) showed that its head skeleton is closely allied to that of *Oryzias* but greatly different from that of *Aplocheilus. Horaichthys,* however, is different from known species of *Oryzias* in having a larger number of the anal fin- rays (about 28 to 32).

In the male, six anterior rays of the anal fin are separated from the rest of the fin and modified into an elaborate male organ (gonopodium). Of six rays the third, fourth and fifth ones are profoundly modified forming the 3- 4-5 complex. (Fig. 2-3). In the female right pelvic fin is usually absent. The genital opening of the female is situated on the left ventral side and is surrounded by genital pads. *Horaichthys* is supposed to have evolved from *Oryzias*, but as the development of the gonopodium in association with the henpecked sexual behavior is so remarkable that Kulkarni (1940) has proposed to erect a new family rank for this fish.

The male appears to be always afraid of the female which on occasions chases him away. At the time of mating, "the male swims below and behind her at a distance of about 2 to 3 cm. He then darts towards her on the left with almost lightning speed. As he approaches his mate he lashes out the gonopodium sideways almost at right angles to his body and strikes its terminal end against her genital opening. The spermatophores are transferred to the female in this momentary contact, and become attached by their distal hooks."

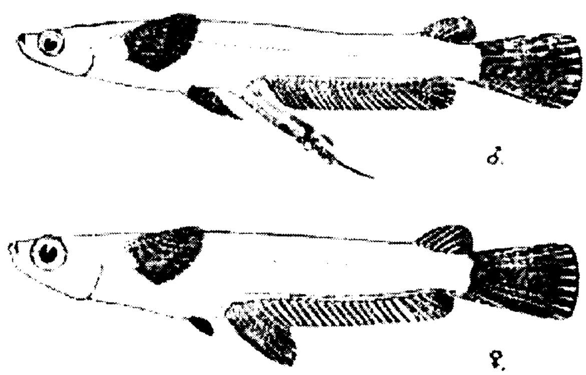


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Fig.2-3. Lateral view of a male and a female specimen of Horaichthys setnai.
x 4 Kulkalni, 1940.
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A special feature of *Horaichthys* is that the testis produces special sperm capsules of spermatophores (2-300 in number) instead of ordinary semi-fluid milt with suspended sperms.

A spermatophore is a tiny hyaline body (0.6 mm long and 0.1 mm thick), the broad part of which contains mass of sperms. At the tapering end, there is a pointed cap with stiff hooks and barb-like structures which point backwards. It is with the aid of these hooks and barbs that the spermatophore get attached near the genital opening of the female.

There is no permanent opening on the spermatophore for the liberation of sperms. Before liberation of sperms, a small bulging appears at the neck of the tapering spermatophore and begins to enlarge. When the protuberance becomes sufficiently large, an opening is formed at its tip by rupture of membrane and sperms are liberated. They swim into the genital pore of the female.

The following is the classification of the new order Atheriniformes by Rosen (1964), representative species being given in parentheses.

```
Suborder Exocoetoidei
   Superfamily Exocoetoidea
        Family Hemiramphidae (Hemiramphus)
        Family Exocoetidae (Exocoetus)
   Superfamily Scomberesocoidea
        Family Belonidae (Ablennus)
        Family Scomberesocidae (Cololabis)
Suborder Cyprinodontoidei
   Superfamily Adrianichthyoidea
        Family Oryziatidae (Oryzias)
        Family Adrianichthyidae (Adrianichthys, Xenopoecilus)
        Family Horaichthyidae (Horaichthys)
   Superfamily Cyprinodontoidea
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        Family Cyprinodontidae (Fundulus, Aplocheilus)
        Family Goodeidae (Goodea)
        Family Jenynsiidae (Jenynsia)
        Family Anablepidae (Anableps)
        Family Poeciliidae (Poecilia, Xiphophorus)
Suborder Atherinoidei
   Superfamily Atherinoidea
        Family Melanotaeniidae
        Family Atherinidae (Atherina)
        Family Isonidae, new family (Iso)
   Superfamily Phallostethoidea
        Family Neostethidae (Neostethus)
        Family Phallostethidae (Phallostethus)
```

The family Oryziatidae

Rosen (1964) erected a new monogeneric family and described the following diagnoses of the family Oryziatidae. Type genus: *Oryzias* Jordan and Snyder, 1906. Diagnoses: The Oryziatidae differ from their closest relatives, the adrianichthyids, in lacking the tremendously enlarged jaws and ethmoideum, in having paired supraoccipital processes (rather than a single median process), and in having the inferior pharyngeal bone distinctly separated (rather than united), and from all cyprinodontoids as follows: autopalatine usually capped by sesamoid bone; pterygoquadrate cartilage forming dorsal process; lower end of premaxilla not hooked or trapezoidal, situated below maxilla rather than between maxilla and dentary bone; first pleural rib on third vertebra; supracleithrum wanting; pelvic bones with upright lateral spurs and not joined midventrally; hypochordal musculature present on caudal fin.

Composition: Rosen listed following seven species of a single genus, *Oryzias: O. latipes* (Temminck and Schlegel), *O. melastigma* (McClelland), *O. celebensis* (Weber), *O. timorensis* (Weber and de Beaufort), *O. javanicus* (Bleeker), *O. curvinotus* (Nichols and Pope), and *O. minutillus* Smith. To these, *O. luzonensis* (Herre and Ablan) may be added. Besides these, Turner (1965) mentioned *O. matenensis* (Aurich), and *O. marmoratus* (Aurich) from the Celebes.

Probably not all these nominal species are valid, since some nominal species are different only in the anal fin-ray frequency.

The genus *Oryzias*

The following is the diagnoses of the genus *Oryzias* described by Jordan and Snyder (1906), basing on *O. latipes* which has previously been known as *Aplocheilus latipes*.

Body elliptical in form, compressed, covered with large scales; mouth small, with two rows of small, simple, pointed teeth; *no teeth on vomer**1; gill-opening not restricted above; intestinal canal short, about as large as body; peritoneum black. Dorsal fin short, inserted above middle of anal; anal very long seventeen to twenty rays; caudal fin truncate. *Sexes similar**2 *except color;* anal fin not modified in the male. *1 Kulkarni(1948) first showed that *os vomer* is absent in *Oryzias melastigma.* *2 Sexual

dimorphism is prominent. See Chap. 8.

The species *Oryzias latipes*

The following description by Oshima (1919) based on a specimen of *Oryzias* latipes collected from Shori, Formosa is cited here as the diagnoses of the species since it is very precise and correct excepting two words starred and daggered.

Head 4 in length (body length divided by head length is 4); depth 4.5; depth of caudal peduncle 9.5; eye 2.5 in head (head length divided by eye diameter is 2.5); interorbital space 2; snout 4; D.6; A.18; P.9; V.5; thirty one scales in a lateral series; five branchiostegals.

Posterior half of the body compressed, becoming broader anteriorly, highest in front of the anal; head flattened; interorbital space broad; snout shorter than the diameter of eye, broadly rounded anteriorly; mouth anterior, transverse; lower jaw slightly projecting, each jaw with two rows of minute pointed teeth, those on the posterior row smaller; vomer*1 smooth; thirteen short, pointed gill-rakers on the first arch; eyes very large, anterior and superior.

Dorsal fin short, on the posterior half of body, its origin above the posterior two thirds of anal, its height equal to the distance between tip of snout and posterior margin of orbit; pectoral inserted on the median line of body; the ventral small, reaching vent; base of the anal very long, its posterior end opposite to that of the dorsal, anterior ray longest; tip of the caudal fin *rounded.**2

Top and sides of head, throat, and chin naked; body covered with cycloid scales, lateral line absent.

Color in formalin pale gray above, lower parts silvery; a black longitudinal streak from the nape to the origin the dorsal; sides of body with a faint dusky stripe along the middle line, top of head dark; the edges of scales dusky; fin-rays of the ventral and anal dotted with minute black spots; all the fins whitish; peritoneum black. Length of body 28 mm.

Habitat: The present species is very common in rice-fields and pools on the island.

*1 Vomer is absent in *Oryzias* in reality.

*2 The caudal fin is almost truncate, strictly, however, it is incipiently lobed.

Change of nomenclature of the medaka

The medaka was first described as *Poecilia latipes* by Temminck and Schlegel in 1846 (Siebold's Fauna Japonica, Poiss., P.224, Pl.102, Fig. 5). GŸnther changed it as *Haplochilus latipes*. Jordan and Snyder (1901) described it as *Aplocheilus latipes* but later they separated it from *Aplocheilus* and erected a new genus *Oryzias*. They regarded *Oryzias* as having no teeth on *vomer**1 while *Aplocheilus* possesses teeth on it.

Myers (1931) placed the medaka in the tribe Aplocheilini of the subfamily Fundulinae in the family Cyprinodontidae. He stated that the chief character of fishes of the tribe is the non-protractile premaxillae. The pectoral fin are set high and pseudobranchiae and vomerine teeth are never present. The species range from Japan and Central China south to Celebes and Timor and west to Southern India. A single genus, *Aplocheilus,* of which *Oryzias* is a synonym*2. Smith, (1945) pointed out that the genus known as Panchax is a synonym of *Aplocheilus* McClelland and *Aplocheilus* Weber and de Beaufort is a synonym of *Oryzias* Jordan and Snyder. He described *Aplocheilus panchax* (Hamilton) and *Oryzias minutillus* n. sp. from Thailand.

According to him, the two genera may be distinguished by the following characters:

```
a.  Upper jaw protractile; mouth moderate size with its corners
     abruptly bent downward; vomer toothed; pseudobranchiae
     present; branchial membranes free from each other and from
     isthmus; pectoral fins with their upper base at or below
     longitudinal axis of body . . . . . . . . . . . . . . . . . . . . . . . . Aploc
b.  Upper jaw not protractile; mouth small with its corners obtusely
     bent downward; vomer toothless; no pseudobranchiae; branchiae
     membranes united across isthmus; pectoral fins with upper base
     well above longitudinal axis of body . . . . . . . . . . . . . . . . . . Oryzia
```

The correct scientific name of the medaka is *Oryzias* latipes (Temminck and Schlegel).

From Jordan and Snyder (1906) onwards, all taxonomists stated that *Oryzias* has toothless vomer while *Aplocheilus* has toothed vomer. Kulkarni (1948) has made a precise osteological study of Indian killifishes and found that vomer is absent in both *Oryzias melastigma* and *Horaichthys setnai* while Aplocheilus lineata possesses toothed vomer.

*1 Vomer is absent in *Oryzias* in reality.
*2 Now, the two genera belong to the different super-families.

Geographical distribution of species belonging to the Genus *Oryzias*

All the species of the genus *Oryzias* are distributed in India, South Asia, the Indo-Australian archipelago and the Far East. Their habitats are widely ranged from tropical, subtropical, and temperate regions as shown in Figure 2-4 and in the following lines.

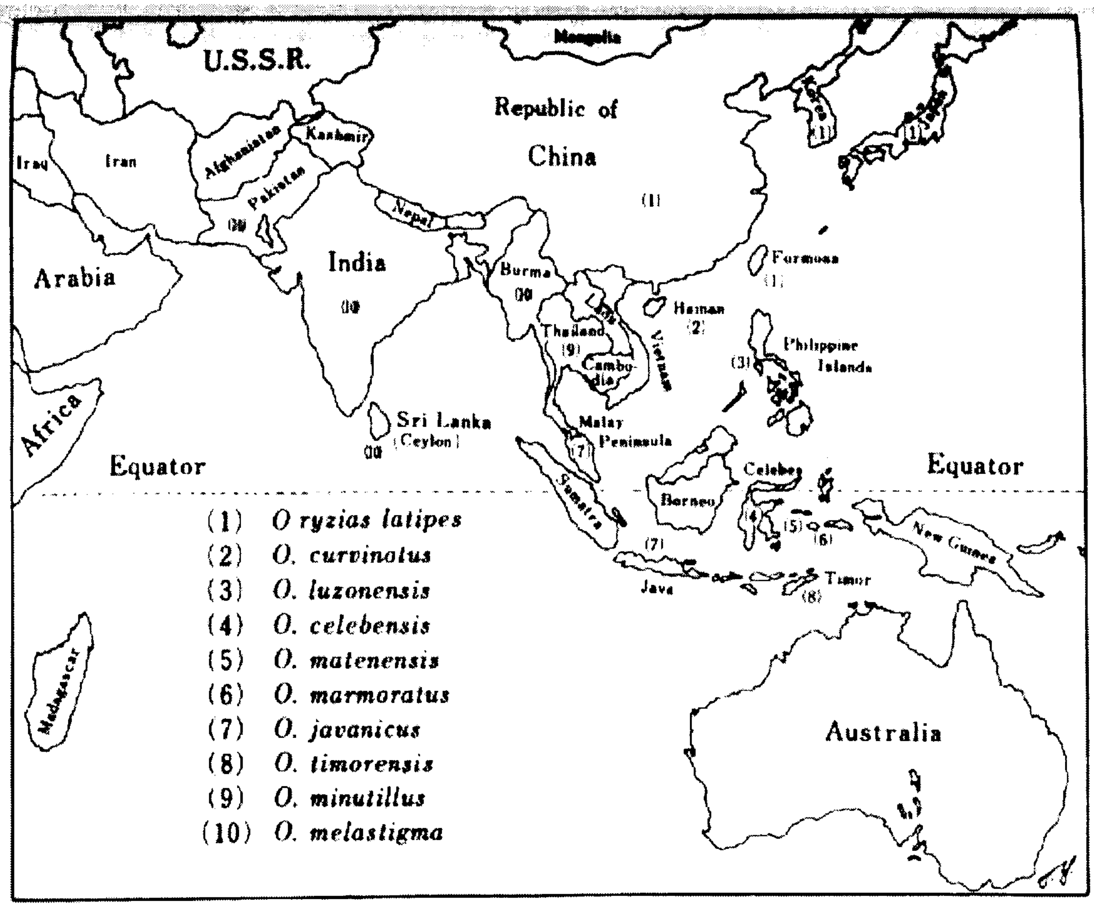

Fig. 2-4. A zoogeographical map showing distribution of species of the Genus *Oryzias*. Original.

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(1) O. *latipes* (Temminck and Shlegel): Japan, Korea, Formosa, and China
(2) O. *curvinotus* (Nicols and Pope): The island of Hainan.
(3) O. *luzonensis* (Herre and Ablan): Luzon in the Philippines.
(4) O. *celebensis* (Weber): The Celebes.
(5) O. *matenensis* (Aurich): The Celebes.
(6) O. *marmoratus* (Aurich): The Celebes.
(7) O. *javanicus* (Bleeker): The Indo-Malaysian archipelago and Malaya.
(8) O. *timorensis* (Weber and de Beaufort): The island of Timor.
(9) O. *minutillus* Smith: Thailand.
(10) O. *melastigma* (McClelland): India, Western Pakistan, and Sri Lanka (Ceylon).

In the main, all the *Oryzias* species are fresh-water fishes. *O. latipes* and *O. melastigma* inhabit both fresh and brackish water. *O. latipes* is so tolerate slinity that it thrives in tide pools in Korea and Kyushu in Japan.

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